

## **Tumour-Specific Animal Proteins**

### **CROSS REFERENCES TO RELATED APPLICATIONS**

This application is a continuation-in-part and claims benefit to U.S. Application  
5 No. 10/226,872, which was filed in the United States Patent and Trademark Office on  
August 23, 2002, which was filed as a continuation-in-part of International Application  
No. PCT/EP01/01779, filed on February 16, 2001, which claims priority of Great Britain  
Patent Application No. 0004269.7, filed February 23, 2000, which claims priority of  
Great Britain Patent Application No. 0009905.1; filed April 20, 2000, which claims  
10 priority of Great Britain Patent Application No. 0021080.7, filed August 25, 2000. This  
application also claims benefit to copending International Application Serial Nos.:  
PCT/EP02/05011, filed 16 May 2001 and PCT/EP02/01649, filed 21 February 2001, the  
contents of which are incorporated herein by reference in their entirety.

### **15 FIELD OF THE INVENTION**

The present invention relates to pharmaceutical compositions and methods for  
inducing an immune response against tumor-related antigens. More specifically, the  
invention relates to polynucleotides, herein referred to as CASB7439 polynucleotides,  
polypeptides encoded thereby (referred to herein as CASB7439 polypeptides),  
20 recombinant materials and methods for their production and use of CASB7439  
polynucleotide and polypeptides in the diagnosis and treatment of cancer.

### **BACKGROUND**

Polypeptides and polynucleotides of the present invention are believed to be  
25 important immunogens for specific prophylactic or therapeutic immunization against  
tumors, because they are specifically expressed or highly over-expressed in tumors  
compared to normal cells and can thus be targeted by antigen-specific immune mechanisms  
leading to the destruction of the tumor cell. They can also be used to diagnose the  
occurrence of tumor cells. Furthermore, their inappropriate expression in certain  
30 circumstances can cause an induction of autoimmune, inappropriate immune responses,  
which could be corrected through appropriate vaccination using the same polypeptides or  
polynucleotides. In this respect the most important biological activities to our purpose are  
the antigenic and immunogenic activities of the polypeptide of the present invention. A

polypeptide of the present invention may also exhibit at least one other biological activity of a CASB7439 polypeptide, which could qualify it as a target for therapeutic or prophylactic intervention different from that linked to the immune response.

## 5 SUMMARY OF THE INVENTION

The present invention relates to a method of treating or preventing cancer in a patient comprising the steps of administering a therapeutically effective amount of a polypeptide comprising an amino acid sequence having at least 70% sequence identity to the amino acid sequence set forth in SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:7, SEQ  
 10 ID NO:10, SEQ ID NO:11, SEQ ID NO:12 or SEQ ID NO:14 over the entire length of SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:7, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12 or SEQ ID NO:14 respectively, wherein the polypeptide may optionally comprise a fusion partner or an affinity tag, wherein administration of said polypeptide to said patient induces an immune response to a tumour antigen. In another aspect, the  
 15 polypeptide is admixed with an adjuvant. In a further aspect, the tumour antigen comprises CASB7439. In another aspect, the patient has or has a potential to contract a cancer comprising colorectal, breast or lung cancer. In another aspect, the polypeptide has at least 95% sequence identity to SEQ ID NO:2.

In another embodiment, the present invention provides a method of inducing an  
 20 immunoresponse to CASB7439 in a human or non-human animal comprising administering a peptide fragment of SEQ ID NO:2 to the human or non-human animal. In one aspect, the peptide fragment is selected from the group consisting of SEQ ID NO: 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, and 33. In another aspect, the peptide fragment further comprises a fusion partner. The peptide fragment or peptide  
 25 fragment with fusion partner may be admixed with an adjuvant.

In another embodiment, the present invention provides a method of manufacturing a medicament for immunotherapeutically treating a patient suffering from or susceptible to cancer comprising expressing a protein in a cell comprising a polynucleotide comprising a nucleotide sequence which has at least 70% sequence identity to the  
 30 nucleotide sequence set forth in SEQ ID NO:1 over the entire length of SEQ ID NO:1. In one aspect, the polynucleotide has at least 95% sequence identity to SEQ ID NO:1. In another aspect, the patient is suffering from a cancer comprising colorectal, breast or lung cancer. In another aspect, the polynucleotide is selected from the group consisting of

- (a) a polynucleotide comprising a nucleotide sequence encoding SEQ ID NO:2;
- (b) the coding region of polynucleotide SEQ ID NO:1; and
- (c) a polynucleotide obtainable by screening an appropriate library under  
 5 stringent hybridization conditions with a labeled probe having the sequence  
 of SEQ ID NO:1 or a fragment thereof, wherein said polynucleotide  
 encodes a polypeptide having similar properties to those fo SEQ ID NO:2.

In another embodiment, the present invention provides a method of manufacturing  
 a medicament comprising a polypeptide which has at least 70% sequence identity to the  
 10 amino acid sequence set forth in SEQ ID NO:2 over the entire length of SEQ ID NO:2 for  
 the manufacture of a medicament for immunotherapeutically treating a patient suffering  
 from or susceptible to cancer. In one aspect, the polypeptide has at least 95% sequence  
 identity to SEQ ID NO:2. In another aspect, the patient is suffering from a cancer  
 comprising colorectal, breast or lung cancer.

15 In yet another embodiment, the present invention provides an immunogenic  
 fragment of CASB7439, wherein the immunogenic fragment is immunologically reactive  
 with an antibody that binds to and/or a T-cell that reacts with or binds to a polypeptide  
 comprising SEQ ID NO:2. In another aspect, a pharmaceutical composition comprising  
 such an immunogenic fragment is provided.

20 In yet another embodiment, the present invention provides polypeptide  
 comprising the amino acid sequence set forth in SEQ ID NO:35. In another aspect,  
 isolated polynucleotides encoding such a polypeptide are provided. In addition,  
 expression vectors comprising such polynucleotides are provided as well as a host cell  
 comprising such expression vectors.

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#### BRIEF DESCRIPTION OF THE FIGURES

Figure 1: Real-time PCR data using the Taqman probe

Figure 2: Real-time PCR expression using Sybr protocol

Figure 3: Coomassie blue stained SDS PAGE of the cell extract from the strain  
 30 expressing CASB7439

Figure 4: Western blot loaded with cell extract from the strain expressing CASB7439.

Figure 5: Coomassie-blue stained SDS-PAGE of CASB7439 after purification. Lanes 1 and 5 represent the molecular weight markers; lanes 2, 3, 4 are respectively loaded with 2  $\mu$ l, 4  $\mu$ l and 6  $\mu$ l of purified protein.

Figure 6: Western blot CASB7439 after purification as revealed by an anti-polyhistidine monoclonal antibody.

Figure 7: IHC results on colon tumour #9476 biospy.

Figure 8: IHC results colon normal mucosa #9476 biospy.

Figure 9: Real-time RT-PCR quantification of CASB7439 transcript on a panel of cell lines, tumor and normal tissues

10 Figure 10: Real-time RT-PCR quantification (3' UTR primers) of CASB7439 transcript on a panel of tumors grown in SCID mice.

Figure 11: Real-time RT-PCR quantification (ORF primers) of CASB7439 transcript on a panel of tumors grown in SCID mice

15 Figure 12: Real-time RT-PCR quantification (3' UTR primers) of CASB7439 transcript on an extended panel of normal and tumors tissues.

Figure 13: Real-time RT-PCR quantification (3' UTR primers) of CASB7439 transcript on an extended panel of normal and tumors tissues

Figure 14: Real-time RT-PCR quantification (3' UTR primers) of CASB7439 transcript on an extended panel of normal and tumors tissues, continued.

20 Figure 15: Design of NS1-CASB7439 protein.

Figure 16: Schematic representation of pMG1 cloning strategy (constructs 1 and 2).

Figure 17: Coomassie blue stained SDS PAGE of the cell extract from the strain expressing CASB7439 construct 1.

25 Figure 18: Western blot loaded with cell extract from the strain expressing CASB7439 construct 1.

Figure 19: Western blot CASB7439 construct 1 after purification (monoclonal anti-NS1 N terminal fragment).

Figure 20: Comparison of Constructs 3 to 5 expression yields. Western blot on CASB7439 constructs 3 to 5 expressed in *E. coli*, revealed with antipeptide SB600 antibody.

Figure 21: Expression of C7439 in HEK 293T Cells by DNA Vaccine Vector pJB76 (pJB16/C7439)

Figure 22: Schematic representation of CASB7439 expressed in a adenoviral live vector

Figure 23: Expression of C7439 in Human Fibroblasts by Recombinant Adenovirus AdC7439

Figure 24: Real-time RT-PCR quantification of CASB7439 transcript on a population of preneoplastic lesions of lung (PLL) patients.

5 Figure 25: Real-time RT-PCR quantification of CASB7439 transcript on a population of early stage lung cancer (ESLC) patients.

Figure 26: Real-time RT-PCR quantification of CASB7439 transcript on a population of late stage lung cancer (LSLC) patients.

10 Figure 27: Real-time RT-PCR quantification of CASB7439 transcript on a panel of normal tissues.

Figure 28: Real-time RT-PCR quantification of CASB7439 transcript on a panel of normal tissues, continued.

Figure 29: Design of NS1-CASB7439 protein

Figure 30: Schematic representation of pMG1 cloning strategy (constructs 1 and 2).

15 Figure 31: Comparison of Constructs 3 to 5 expression yields. Western blot on CASB7439 constructs 3 to 5 expressed in *E. coli*, revealed with antipeptide SB600 antibody.

Figure 32: CASB7439 peptide 1 immunization of rabbit SB598: graphical view of ELISA read-outs (Antibody titers relative to CASB7439 peptide 1, straight line; antibody  
20 titers relative to KLH carrier, discontinuous line)

Figure 33: CASB7439 peptide 1 immunization of rabbit SB599: graphical view of ELISA read-outs (Antibody titers relative to CASB7439 peptide 1, straight line; antibody titers relative to KLH carrier, discontinuous line).

Figure 34: CASB7439 peptide 2 immunization of rabbit SB600: graphical view of  
25 ELISA read-outs (Antibody titers relative to CASB7439 peptide 2, straight line; antibody titers relative to KLH carrier, discontinuous line).

Figure 35: CASB7439 peptide 2 immunization of rabbit SB601: graphical view of ELISA read-outs (Antibody titers relative to CASB7439 peptide 2, straight line; antibody titers relative to KLH carrier, discontinuous line).

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## DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to pharmaceutical compositions and methods for inducing an immune response against tumor-related antigens. More specifically, the

invention relates to polynucleotides, herein referred to as CASB7439 polynucleotides, polypeptides encoded thereby (referred to herein as CASB7439 polypeptides), recombinant materials and methods for their production. In another aspect, the invention relates to methods for using such polypeptides and polynucleotides, including the treatment  
5 of cancer, more particularly colorectal cancer, breast cancer, and lung cancer and autoimmune diseases and other related conditions. In another aspect, the invention relates to pharmaceutical compositions containing CASB7439 polypeptides and polynucleotides, to methods of manufacture of such compositions and to their use in medicine. In a further aspect, the invention relates to methods for identifying agonists and antagonists/inhibitors  
10 using the materials provided by the invention, and treating conditions associated with CASB7439 polypeptide imbalance with the identified compounds. In a still further aspect, the invention relates to diagnostic assays for detecting diseases associated with inappropriate CASB7439 polypeptide activity or levels.

In a first aspect, the present invention relates to CASB7439 polypeptides. Such  
15 peptides include isolated polypeptides, comprising an amino acid sequence which has at least 70% identity, or at least 80% identity, or at least 90% identity, or at least 95% identity, or at least 97-99% identity, to that of SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:7, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12 or SEQ ID NO:14 over the entire length of SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:7, SEQ ID NO:10, SEQ ID NO:11,  
20 SEQ ID NO:12 or SEQ ID NO:14 respectively. Such polypeptides include those comprising the amino acid of SEQ ID NO:3, SEQ ID NO:7, SEQ ID NO:10 and SEQ ID NO:11.

Further peptides of the present invention include isolated polypeptides, in which the amino acid sequence has at least 70% identity, or at least 80% identity, or at least 90%  
25 identity, or at least 95% identity, or at least 97-99% identity, to the amino acid sequence of SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:7, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12 or SEQ ID NO:14 over the entire length of SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:7, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12 or SEQ ID NO:14, respectively. Such polypeptides include the polypeptides of SEQ ID NO:3, SEQ ID  
30 NO:7, SEQ ID NO:10 and SEQ ID NO:11.

The aforementioned polypeptides may be recombinantly produced. The polypeptides according to the invention may be purified, and are substantially free of any other proteins or contaminating host-originating material.

Further peptides of the present invention include isolated polypeptides encoded by a polynucleotide comprising the sequence contained in SEQ ID NO:1.

The invention also provides an immunogenic fragment of a CASB7439 polypeptide, that is a contiguous portion of the CASB7439 polypeptide which has the same or similar  
 5 immunogenic properties to the polypeptide comprising the amino acid sequence of SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:7, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12 or SEQ ID NO:14. That is to say, the fragment (if necessary when coupled to a carrier or as part of a larger fusion protein) is capable of raising an immune response which recognizes the CASB7439 polypeptide. Such an immunogenic fragment may include, for example, the  
 10 CASB7439 polypeptide lacking an N-terminal leader sequence, a transmembrane domain or a C-terminal anchor domain. The immunogenic fragment of CASB7439 according to the invention may comprises substantially all of the extracellular domain of a polypeptide which has at least 70% identity, or at least 80% identity, or at least 90% identity, or at least 95% identity, or at least 97-99% identity, to that of SEQ ID NO:2, SEQ ID NO:3, SEQ ID  
 15 NO:7, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12 or SEQ ID NO:14 over the entire length of SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:7, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12 or SEQ ID NO:14, respectively. An immunogenic fragment according to the invention may comprise at least one epitope.

Peptide fragments incorporating an epitope of CASB7439 typically will comprise  
 20 at least 7, or 9 or 10 contiguous amino acids from SEQ ID NO:2. Epitopes also include those shown in SEQ ID NO:16 to SEQ ID NO:33.

Peptides that incorporate these epitopes form one aspect of the present invention. Mimotopes which have the same characteristics as these epitopes, and immunogens comprising such mimotopes which generate an immune response which cross-react with  
 25 an epitope in the context of the CASB7439 molecule, also form part of the present invention.

The present invention, therefore, includes isolated peptides encompassing these epitopes themselves, and any mimotope thereof. The meaning of mimotope is defined as an entity which is sufficiently similar to the native CASB7439 epitope so as to be capable  
 30 of being recognized by antibodies which recognize the native molecule; (Gheysen, H.M., *et al.*, 1986, Synthetic peptides as antigens. Wiley, Chichester, Ciba foundation symposium 119, p130-149; Gheysen, H.M., 1986, Molecular Immunology, 23,7, 709-

715); or are capable of raising antibodies, when coupled to a suitable carrier, which antibodies cross-react with the native molecule.

Peptide mimotopes of the above-identified epitopes may be designed for a particular purpose by addition, deletion or substitution of elected amino acids. Thus, the peptides of the present invention may be modified for the purposes of ease of conjugation to a protein carrier. For example, it may be desirable for some chemical conjugation methods to include a terminal cysteine to the epitope. In addition it may be desirable for peptides conjugated to a protein carrier to include a hydrophobic terminus distal from the conjugated terminus of the peptide, such that the free unconjugated end of the peptide remains associated with the surface of the carrier protein. This reduces the conformational degrees of freedom of the peptide, and thus increases the probability that the peptide is presented in a conformation which most closely resembles that of the peptide as found in the context of the whole molecule. For example, the peptides may be altered to have an N-terminal cysteine and a C-terminal hydrophobic amidated tail. Alternatively, the addition or substitution of a D-stereoisomer form of one or more of the amino acids may be performed to create a beneficial derivative, for example to enhance stability of the peptide. Those skilled in the art will realise that such modified peptides, or mimotopes, could be a wholly or partly non-peptide mimotope wherein the constituent residues are not necessarily confined to the 20 naturally occurring amino acids. In addition, these may be cyclized by techniques known in the art to constrain the peptide into a conformation that closely resembles its shape when the peptide sequence is in the context of the whole molecule. Cyclizing a peptide may comprise the addition of a pair of cysteine residues to allow the formation of a disulphide bridge.

Further, those skilled in the art will realise that mimotopes or immunogens of the present invention may be larger than the above-identified epitopes, and as such may comprise the sequences disclosed herein. Accordingly, the mimotopes of the present invention may consist of addition of N and/or C terminal extensions of a number of other natural residues at one or both ends. The peptide mimotopes may also be retro sequences of the natural sequences, in that the sequence orientation is reversed; or alternatively the sequences may be entirely or at least in part comprised of D-stereo isomer amino acids (inverso sequences). Also, the peptide sequences may be retro-inverso in character, in that the sequence orientation is reversed and the amino acids are of the D-stereoisomer



form. Such retro or retro-inverso peptides have the advantage of being non-self, and as such may overcome problems of self-tolerance in the immune system.

Alternatively, peptide mimotopes may be identified using antibodies which are capable themselves of binding to the epitopes of the present invention using techniques  
 5 such as phage display technology (EP 0 552 267 B1). This technique, generates a large number of peptide sequences which mimic the structure of the native peptides and are, therefore, capable of binding to anti-native peptide antibodies, but may not necessarily themselves share significant sequence homology to the native peptide. This approach may have significant advantages by allowing the possibility of identifying a peptide with  
 10 enhanced immunogenic properties, or may overcome any potential self-antigen tolerance problems which may be associated with the use of the native peptide sequence. Additionally this technique allows the identification of a recognition pattern for each native-peptide in terms of its shared chemical properties amongst recognised mimotope sequences.

15 The covalent coupling of the peptide to the immunogenic carrier can be carried out in a manner well known in the art. Thus, for example, for direct covalent coupling it is possible to utilise a carbodiimide, glutaraldehyde or (N-[ $\gamma$ -maleimidobutyryloxy] succinimide ester, utilising common commercially available heterobifunctional linkers such as CDAP and SPDP (using manufacturers instructions). After the coupling reaction,  
 20 the immunogen can easily be isolated and purified by means of a dialysis method, a gel filtration method, a fractionation method etc.

The types of carriers used in the immunogens of the present invention will be readily known to the man skilled in the art. The function of the carrier is to provide cytokine help in order to help induce an immune response against the peptide. A non-  
 25 exhaustive list of carriers which may be used in the present invention include: Keyhole limpet Haemocyanin (KLH), serum albumins such as bovine serum albumin (BSA), inactivated bacterial toxins such as tetanus or diphtheria toxins (TT and DT), or recombinant fragments thereof (for example, Domain 1 of Fragment C of TT, or the translocation domain of DT), or the purified protein derivative of tuberculin (PPD).  
 30 Alternatively the mimotopes or epitopes may be directly conjugated to liposome carriers, which may additionally comprise immunogens capable of providing T-cell help. The ratio of mimotopes to carrier is in the order of 1:1 to 20:1, and each carrier may carry between 3-15 peptides.

In one embodiment of the invention a carrier is Protein D from *Haemophilus influenzae* (EP 0 594 610 B1). Protein D is an IgD-binding protein from *Haemophilus influenzae* and has been patented by Forsgren (WO 91/18926, granted EP 0 594 610 B1). In some circumstances, for example in recombinant immunogen expression systems it may be desirable to use fragments of protein D, for example Protein D 1/3<sup>rd</sup> (comprising the N-terminal 100-110 amino acids of protein D (GB 9717953.5)).

Another method of presenting the peptides of the present invention is in the context of a recombinant fusion molecule. For example, EP 0 421 635 B describes the use of chimaeric hepadnavirus core antigen particles to present foreign peptide sequences in a virus-like particle. As such, immunogens of the present invention may comprise peptides presented in chimaeric particles consisting of hepatitis B core antigen. Additionally, the recombinant fusion proteins may comprise the mimotopes of the present invention and a carrier protein, such as NS1 of the influenza virus. For any recombinantly expressed protein which forms part of the present invention, the nucleic acid which encodes said immunogen also forms an aspect of the present invention.

Peptides used in the present invention can be readily synthesised by solid phase procedures well known in the art. Suitable syntheses may be performed by utilising "T-boc" or "F-moc" procedures. Cyclic peptides can be synthesised by the solid phase procedure employing the well-known "F-moc" procedure and polyamide resin in the fully automated apparatus. Alternatively, those skilled in the art will know the necessary laboratory procedures to perform the process manually. Techniques and procedures for solid phase synthesis are described in 'Solid Phase Peptide Synthesis: A Practical Approach' by E. Atherton and R.C. Sheppard, published by IRL at Oxford University Press (1989). Alternatively, the peptides may be produced by recombinant methods, including expressing nucleic acid molecules encoding the mimotopes in a bacterial or mammalian cell line, followed by purification of the expressed mimotope. Techniques for recombinant expression of peptides and proteins are known in the art, and are described in Maniatis, T., Fritsch, E.F. and Sambrook *et al.*, *Molecular cloning, a laboratory manual*, 2nd Ed.; Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (1989).

In a further embodiment of the invention is provided a method of producing a polypeptide as described herein. The process of the invention may be performed by conventional recombinant techniques such as described in Maniatis *et al.*, *Molecular*

Cloning - A Laboratory Manual; Cold Spring Harbor, 1982-1989. Accordingly there is provided a process for producing a polypeptide according to the invention, comprising culturing a host cell under conditions sufficient for the production of said polypeptide and recovering the polypeptide from the culture medium. In particular, the process of the invention may comprise the steps of:

- i) preparing a replicable or integrating expression vector capable, in a host cell, of expressing a DNA polymer comprising a nucleotide sequence that encodes the protein or an immunogenic derivative thereof;
- ii) transforming a host cell with said vector;
- ii) culturing said transformed host cell under conditions permitting expression of said DNA polymer to produce said protein; and
- iv) recovering said protein.

The polypeptides or immunogenic fragment of the invention may be in the form of the "mature" protein or may be a part of a larger protein such as a precursor or a fusion protein. It is often advantageous to include an additional amino acid sequence which contains secretory or leader sequences, pro-sequences, sequences which aid in purification such as multiple histidine residues, or an additional sequence for stability during recombinant production. Furthermore, addition of exogenous polypeptide or lipid tail or polynucleotide sequences to increase the immunogenic potential of the final molecule is also considered.

In one aspect, the invention relates to genetically engineered soluble fusion proteins comprising a polypeptide of the present invention, or a fragment thereof, and various portions of the constant regions of heavy or light chains of immunoglobulins of various subclasses (IgG, IgM, IgA, IgE). An immunoglobulin may include the constant part of the heavy chain of human IgG, particularly IgG1, where fusion takes place at the hinge region. In a particular embodiment, the Fc part can be removed simply by incorporation of a cleavage sequence which can be cleaved with blood clotting factor Xa. Furthermore, this invention relates to processes for the preparation of these fusion proteins by genetic engineering, and to the use thereof for drug screening, diagnosis and therapy. Another aspect of the invention relates to the use of a polypeptide or a polynucleotide in the manufacture of a vaccine for immunotherapeutically treating a patient suffering from or susceptible to carcinoma, especially colon cancer or other colon-

associated tumours or diseases. A further aspect of the invention also relates to polynucleotides encoding such fusion proteins. Examples of fusion protein technology can be found in International Patent Application Nos. WO94/29458 and WO94/22914.

The proteins may be chemically conjugated, or expressed as recombinant fusion proteins allowing increased levels to be produced in an expression system as compared to non-fused protein. The fusion partner may assist in providing T helper epitopes (immunological fusion partner), including T helper epitopes recognised by humans, or assist in expressing the protein (expression enhancer) at higher yields than the native recombinant protein. The fusion partner may be both an immunological fusion partner and expression enhancing partner.

Fusion partners include protein D from *Haemophilus influenza* B and the non-structural protein from influenzae virus, NS1 (hemagglutinin). Another immunological fusion partner is the protein known as LYTA. The C terminal portion of the molecule may be used. Lyta is derived from *Streptococcus pneumoniae* which synthesize an N-acetyl-L-alanine amidase, amidase LYTA, (coded by the *lytA* gene {Gene, 43 (1986) page 265-272} an autolysin that specifically degrades certain bonds in the peptidoglycan backbone. The C-terminal domain of the LYTA' protein is responsible for the affinity to the choline or to some choline analogues such as DEAE. This property has been exploited for the development of *E. coli* C-LYTA expressing plasmids useful for expression of fusion proteins. Purification of hybrid proteins containing the C-LYTA fragment at its amino terminus has been described {Biotechnology: 10, (1992) page 795-798}. It is possible to use the repeat portion of the Lyta molecule found in the C terminal end starting at residue 178, for example residues 188 - 305.

The present invention also includes xenogeneic forms (also termed ortholog forms) of the aforementioned polypeptides, said xenogeneic forms referring to an antigen having substantial sequence identity to the human antigen (also termed autologous antigen) which serves as a reference antigen but which is derived from a different non-human species. In this context the substantial identity refers to concordance of an amino acid sequence with another amino acid sequence or of a polynucleotide sequence with another polynucleotide sequence when such sequence are arranged in a best fit alignment in any of a number of sequence alignment proteins known in the art. By substantial identity is meant at least 70-95%, or at least 85-95%, or at least 90%-95%, sequence identity between the compared sequences. Therefore according to the invention the

xenogeneic CASB7439 polypeptide will be a CASB7439 polypeptide which is xenogeneic with respect to human CASB7439, in other words which is isolated from a species other than human. In one embodiment, the polypeptide is isolated from mouse, rat, pig, or rhesus monkey. Accordingly the present invention also provides a method of inducing an immune response against human CASB7439 having an amino acid sequence as set forth in any of the sequences SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:7, SEQ ID NO:10 or SEQ ID NO:11 in a human, comprising administering to the subject an effective dosage of a composition comprising a xenogeneic form of said human CASB7439 as described herein. Another embodiment is a method of inducing an immune response against human CASB7439 using the xenogeneic CASB7439 isolated from mouse, rat, pig or rhesus monkey. Another method of inducing an immune response according to the present invention includes using an antigen composition including a live viral expression system which expresses said xenogeneic antigen. The xenogeneic CASB7439 polypeptide may have the sequence set forth in SEQ ID N°12 (mouse) or in SEQ ID N°14 (rat).

The isolated xenogeneic CASB7439 polypeptide will generally share substantial sequence similarity, and include isolated polypeptides comprising an amino acid sequence which has at least 70% identity, or at least 80% identity, or at least 90% identity, or at least 95% identity, or at least 97-99% identity, to that of SEQ ID NO:12 or SEQ ID N°14 over the entire length of SEQ ID NO:12 or SEQ ID N°14. Accordingly the xenogeneic polypeptide may comprise an immunogenic fragment of the polypeptide of SEQ ID NO:12 or SEQ ID NO:14 in which the immunogenic activity of the immunogenic fragment is substantially the same as the polypeptide of SEQ ID NO:12 or SEQ ID NO:14. In addition the xenogeneic CASB7439 polypeptide can be a fragment of at least about 20 consecutive amino acids, or about 30, or about 50, or about 100, or 150 contiguous amino acids selected from the amino acid sequences as shown in SEQ ID NO:12 or in SEQ ID N°14. More particularly xenogeneic CASB7439 fragments will retain some functional property, which may be immunological activity, of the larger molecule set forth in SEQ ID NO:12 or in SEQ ID N°14, and are useful in the methods described herein (e.g. in pharmaceutical and vaccine compositions, in diagnostics, etc.). In particular the fragments will be able to generate an immune response against the human counterpart, such as the generation of cross-reactive antibodies which react with the autologous human form of CASB7439 as set forth in any of the SEQ ID NO: 2. In a

specific embodiment, the xenogeneic polypeptide of the invention may be part of a larger fusion, comprising the xenogeneic CASB7439 polypeptide or fragment thereof and a heterologous protein or part of a protein acting as a fusion partner as described hereabove.

5           The present invention also includes variants of the aforementioned polypeptides, that is polypeptides that vary from the referents by conservative amino acid substitutions, whereby a residue is substituted by another with like characteristics. Typical such substitutions are among Ala, Val, Leu and Ile; among Ser and Thr; among the acidic residues Asp and Glu; among Asn and Gln; and among the basic residues Lys and Arg; or  
10   aromatic residues Phe and Tyr. Variants in which several, 5-10, 1-5, 1-3, 1-2 or 1 amino acids are substituted, deleted, or added in any combination are also included within the invention.

          Polypeptides of the present invention can be prepared in any suitable manner. Such polypeptides include isolated naturally occurring polypeptides, recombinantly  
15   produced polypeptides, synthetically produced polypeptides, or polypeptides produced by a combination of these methods. Means for preparing such polypeptides are well understood in the art.

          In a further aspect, the present invention relates to CASB7439 polynucleotides. Such polynucleotides include isolated polynucleotides comprising a nucleotide sequence  
20   encoding a polypeptide which has at least 70% identity, or at least 80% identity, or at least 90% identity, or at least 95% identity, to the amino acid sequence of SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:7, SEQ ID NO:10 or SEQ ID NO:11, over the entire length of SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:7, SEQ ID NO:10 or SEQ ID NO:11  
25   respectively. In this regard, encoded polypeptides which have at least 97% or at least 98-99% identity or at least 99% identity are considered part of the invention.

          Further polynucleotides of the present invention include isolated polynucleotides comprising a nucleotide sequence that has at least 70% identity, or at least 80% identity, or at least 90% identity, or at least 95% identity, to a nucleotide sequence encoding a polypeptide of SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:7, SEQ ID NO:10, or SEQ ID  
30   NO:11, over the entire coding region. In this regard, polynucleotides which have at least 97% or at least 98-99% identity or at least 99% identity are also included.

          Further polynucleotides of the present invention include isolated polynucleotides comprising a nucleotide sequence which has at least 70% identity, or at least 80%

identity, or at least 90% identity, or at least 95% identity, to SEQ ID NO:1, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:8 or SEQ ID NO:9, over the entire length of said sequences, or to the coding sequence of SEQ ID NO:1, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:8 or SEQ ID NO:9 over the entire length of said

5 coding sequence of SEQ ID NO:1, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:8 or SEQ ID NO:9. In this regard, polynucleotides which have at least 97% identity or at least 98-99% identity or at least 99% identity are also included within the invention. Such polynucleotides include a polynucleotide comprising the polynucleotide of SEQ ID NO:1, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:8 or SEQ ID NO:9 as

10 well as the polynucleotide of SEQ ID NO:1, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:9 or the coding region of SEQ ID NO:1, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:8 or SEQ ID NO:9.

The present invention also provides a nucleic acid encoding the aforementioned xenogeneic proteins of the present invention and their use in medicine. In another

15 embodiment, the xenogeneic CASB7439 polynucleotide for use in pharmaceutical compositions has the sequence set forth in SEQ ID N°13 (mouse) or in SEQ ID N°15 (rat). The isolated xenogeneic CASB7439 polynucleotides according to the invention may be single-stranded (coding or antisense) or double-stranded, and may be DNA (genomic, cDNA or synthetic) or RNA molecules. Additional coding or non-coding

20 sequences may, but need not, be present within a polynucleotide of the present invention. In other related embodiments, the present invention provides polynucleotide variants having substantial identity to the sequences disclosed herein in SEQ ID N°13 or in SEQ ID N°15, for example those comprising at least 70% sequence identity, or at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% or higher, sequence identity compared to

25 a polynucleotide sequence of this invention using the methods described herein, (e.g., BLAST analysis using standard parameters). In a related embodiment, the isolated xenogeneic polynucleotide of the invention will comprise a nucleotide sequence encoding a polypeptide that has at least 90%, or 95% and above, identity to the amino acid sequence of SEQ ID NO:12 or of SEQ ID NO:14, over the entire length of SEQ ID

30 NO:12 or of SEQ ID NO:14; or a nucleotide sequence complementary to said isolated polynucleotide.

The invention also provides polynucleotides which are complementary to all the above described polynucleotides.

Said polynucleotides can be inserted in a suitable plasmid, recombinant microorganism vector or a recombinant live microorganism and used for immunization (see for example Wolff *et. al.*, *Science* 247:1465-1468 (1990); Corr *et. al.*, *J. Exp. Med.* 184:1555-1560 (1996); Doe *et. al.*, *Proc. Natl. Acad. Sci.* 93:8578-8583 (1996)).

5 Accordingly there is provided in the present invention an expression vector or recombinant live microorganism comprising said polynucleotides as hereabove defined.

The invention also provides a fragment of a CASB7439 polynucleotide which when administered to a subject has the same immunogenic properties as the polynucleotide of SEQ ID NO:1, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID  
10 NO:8, SEQ ID NO:9, SEQ ID NO:13 or SEQ ID NO:15.

The invention also provides a polynucleotide encoding an immunological fragment of a CASB7439 polypeptide as hereinbefore defined.

The fragments have a level of immunogenic activity of at least about 50%, or at least about 70% or at least about 90% of the level of immunogenic activity of a  
15 polypeptide sequence set forth in SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:7, SEQ ID NO:10 or SEQ ID NO:11, SEQ ID NO:12 or SEQ ID NO:14 or a polypeptide sequence encoded by a polynucleotide sequence set forth in SEQ ID NO:1, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:13 or SEQ ID NO:15.

The polypeptide fragments according to the invention may comprise at least about  
20 5, 10, 15, 20, 25, 50, or 100 contiguous amino acids, or more, including all intermediate lengths, of a polypeptide composition set forth herein, such as those set forth in SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:7, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12 or SEQ ID NO:14, or those encoded by a polynucleotide sequence set forth in a sequence of SEQ ID NO:1, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:8, SEQ ID  
25 NO:9, SEQ ID NO:13 or SEQ ID NO:15.

The nucleotide sequence of SEQ ID NO:1 is a cDNA sequence which comprises a polypeptide encoding sequence (nucleotide 545 to 1126) encoding a polypeptide of 193 amino acids, the polypeptide of SEQ ID NO:2. The nucleotide sequence encoding the polypeptide of SEQ ID NO:2 may be identical to the polypeptide encoding sequence  
30 contained in SEQ ID NO:1 or it may be a sequence other than the one contained in SEQ ID NO:1, which, as a result of the redundancy (degeneracy) of the genetic code, also encodes the polypeptide of SEQ ID NO:2. The polypeptide of the SEQ ID NO:2 is structurally related to other proteins of the achaete scute family, and is also named



“human Achaete Scute homologue 2” (HASH2) (accession number NP\_005161 and AAB86993).

Human Achaete Scute homologue 2 (HASH2) gene, officially designated human ASCL2 (Achaete Scute complex like 2) is a homologue of the *Drosophila* Achaete and Scute genes. Human ASCL2 is expressed in the extravillous trophoblasts of the developing placenta only, and maps on chromosome 11p15 close to IGF2 and H19. The mouse achaete-scute homolog-2 gene (MASH2) encodes a transcription factor playing a role in the development of the trophoblast. The Mash2 gene is paternally imprinted in the mouse, and the lack of human ASCL2 expression in non-malignant hydatidiform (androgenetic) moles indicates that human Ascl2 is also imprinted in man.

Ascl2 genes are members of the basic helix-loop-helix (BHLH) family of transcription factors. They activate transcription by binding to the E box (5'-CANNTG-3'). Dimerization with other BHLH proteins is required for efficient DNA binding. They are involved in the determination of the neuronal precursors in the peripheral nervous system and the central nervous system in *drosophila melanogaster*, and probably in mammals as well.

The complementary strand of the nucleotide sequence of SEQ ID NO:1 is the polynucleotide sequence of SEQ ID NO:6. This strand also comprises two other polypeptide encoding sequences. The first polypeptide encoding sequence (nucleotide 1184 to 399 of SEQ ID:1, nucleotide 608 to 1393 of SEQ ID NO:6) encodes a polypeptide of 262 amino acids, the polypeptide of SEQ ID NO:3. The second polypeptide encoding sequence (nucleotide 840 to 262 of SEQ ID NO:1, nucleotide 952 to 1530 of SEQ ID NO:6) encodes a polypeptide of 193 amino acids, the polypeptide of SEQ ID NO: 11. The nucleotide sequence encoding the polypeptides of SEQ ID NO:3 and SEQ ID NO:11 may be identical to the polypeptides encoding sequence contained in SEQ ID NO: 6 or it may be a sequence other than the one contained in SEQ ID NO: 6, which, as a result of the redundancy (degeneracy) of the genetic code, also encodes the polypeptides of SEQ ID NO:3 and 11. The polypeptide of the SEQ ID NO:3 is structurally related to other proteins of the splicing coactivator protein family, having homology and/or structural similarity with homo sapiens splicing coactivator subunit srm300 (genbank accession AAF21439). The polypeptide of SEQ ID NO:11 is not related to any known protein. Polypeptide sequences as set forth in SEQ ID NO:3 and

SEQ ID NO:11, and polynucleotide sequences as set forth in SEQ ID NO:6 are novel and also form part of the invention.

Polypeptides and polynucleotides of the present invention are expected to have, *inter alia*, similar biological functions/properties to their homologous polypeptides and polynucleotides. Furthermore, polypeptides, immunological fragments and polynucleotides of the present invention have at least one activity of either SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3 or SEQ ID NO:11 as appropriate.

The present invention also relates to partial or other incomplete polynucleotide and polypeptide sequences which were first identified prior to the determination of the corresponding full length sequences of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, and SEQ ID NO:11.

Accordingly, in a further aspect, the present invention provides for an isolated polynucleotide which:

- (a) comprises a nucleotide sequence which has at least 70% identity, or 80% identity, or at least 90% identity, or at least 95% identity, or at least 97-99% identity to SEQ ID NO:4 and 5 over the entire length of SEQ ID NO 4 and 5;
- (b) has a nucleotide sequence which has at least 70% identity, or at least 80% identity, or at least 90% identity, or at least 95% identity, or at least 97-99% identity, to SEQ ID NO:1 or SEQ ID NO:6 over the entire length of SEQ ID NO:4 and SEQ ID NO:5 respectively;
- (c) the polynucleotide of SEQ ID NO:4 and SEQ ID NO:5; or
- (d) a nucleotide sequence encoding a polypeptide which has at least 70% identity, or at least 80% identity, or at least 90% identity, or at least 95% identity, or at least 97-99% identity, to the amino acid sequence of SEQ ID NO:2 and SEQ ID NO:7 respectively, over the entire length of SEQ ID NO:2 and 7, as well as the polynucleotides of SEQ ID NO:4 and 5.

The present invention further provides for a polypeptide which:

- (a) comprises an amino acid sequence which has at least 70% identity, or at least 80% identity, or at least 90% identity, or at least 95% identity, or at least 97-99% identity, to that of SEQ ID NO:2 and 7 over the entire length of SEQ ID NO:2 or 7;

(b) has an amino acid sequence which is at least 70% identity, or at least 80% identity, or at least 90% identity, or at least 95% identity, or at least 97-99% identity, to the amino acid sequence of SEQ ID NO:2 or 7 over the entire length of SEQ ID NO:2 or 7;

5 (c) comprises the amino acid of SEQ ID NO:2 or 7; and

(d) is the polypeptide of SEQ ID NO: 7;

as well as polypeptides encoded by a polynucleotide comprising the sequence contained in SEQ ID NO:4 and 5.

10 Polynucleotides of the present invention may be obtained, using standard cloning and screening techniques, from a cDNA library derived from mRNA in cells of human colon cancer, (for example Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual*, 2nd Ed., Cold Spring harbor Laboratory Press, Cold Spring harbor, N.Y. (1989)). Polynucleotides of the invention can also be obtained from natural sources such as  
15 genomic DNA libraries or can be synthesized using well known and commercially available techniques.

When polynucleotides of the present invention are used for the recombinant production of polypeptides of the present invention, the polynucleotide may include the coding sequence for the mature polypeptide, by itself; or the coding sequence for the  
20 mature polypeptide in reading frame with other coding sequences, such as those encoding a leader or secretory sequence, a pre-, or pro- or prepro- protein sequence, or other fusion peptide portions. For example, a marker sequence which facilitates purification of the fused polypeptide can be encoded. In certain embodiments of this aspect of the invention, the marker sequence is a hexa-histidine peptide, as provided in the pQE vector  
25 (Qiagen, Inc.) and described in Gentz *et al.*, *Proc Natl Acad Sci USA* (1989) 86:821-824, or is an HA tag. The polynucleotide may also contain non-coding 5' and 3' sequences, such as transcribed, non-translated sequences, splicing and polyadenylation signals, ribosome binding sites and sequences that stabilize mRNA.

Further embodiments of the present invention include polynucleotides encoding  
30 polypeptide variants which comprise the amino acid sequence of SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:7, SEQ ID NO:11, SEQ ID NO:13 or SEQ ID NO:15 and in which several, for instance from 5 to 10, 1 to 5, 1 to 3, 1 to 2 or 1, amino acid residues are substituted, deleted or added, in any combination.

Polynucleotides which are identical or sufficiently identical to a nucleotide sequence contained in SEQ ID NO:1 or in SEQ ID NO:6, may be used as hybridization probes for cDNA and genomic DNA or as primers for a nucleic acid amplification (PCR) reaction, to isolate full-length cDNAs and genomic clones encoding polypeptides of the present invention and to isolate cDNA and genomic clones of other genes (including genes encoding paralogs from human sources and orthologs and paralogs from species other than human) that have a high sequence similarity to SEQ ID NO:1 or to SEQ ID NO:6. Typically these nucleotide sequences are 70% identical, 80% identical, 90% identical, or 95% identical to that of the referent. The probes or primers will generally comprise at least 15 nucleotides, or at least 30 nucleotides and may have at least 50 nucleotides. Particularly, probes may have between 30 and 50 nucleotides. Particularly primers may have between 20 and 25 nucleotides. In particular, polypeptides or polynucleotides derived from sequences from homologous animal origin could be used as immunogens to obtain a cross-reactive immune response to the human gene.

A polynucleotide encoding a polypeptide of the present invention, including homologs from species other than human, may be obtained by a process which comprises the steps of screening an appropriate library under stringent hybridization conditions with a labeled probe having the sequence of SEQ ID NO: 1 or SEQ ID NO:6 or a fragment thereof; and isolating full-length cDNA and genomic clones containing said polynucleotide sequence. Such hybridization techniques are well known to the skilled artisan. Stringent hybridization conditions may include overnight incubation at 42°C in a solution comprising: 50% formamide, 5xSSC (150mM NaCl, 15mM trisodium citrate), 50 mM sodium phosphate (pH7.6), 5x Denhardt's solution, 10 % dextran sulfate, and 20 microgram/ml denatured, sheared salmon sperm DNA; followed by washing the filters in 0.1x SSC at about 65°C. Thus the present invention also includes polynucleotides obtainable by screening an appropriate library under stringent hybridization conditions with a labeled probe having the sequence of SEQ ID NO:1 or SEQ ID NO:6 or a fragment thereof.

The skilled artisan will appreciate that, in many cases, an isolated cDNA sequence will be incomplete, in that the region coding for the polypeptide is short at the 5' end of the cDNA.

There are several methods available and well known to those skilled in the art to obtain full-length cDNAs, or extend short cDNAs, for example those based on the

method of Rapid Amplification of cDNA ends (RACE) (see, for example, Frohman *et al.*, PNAS USA 85, 8998-9002, 1988). Recent modifications of the technique, exemplified by the Marathon™ technology (Clontech Laboratories Inc.) for example, have significantly simplified the search for longer cDNAs. In the Marathon™ technology, cDNAs have been prepared from mRNA extracted from a chosen tissue and an 'adaptor' sequence ligated onto each end. Nucleic acid amplification (PCR) is then carried out to amplify the 'missing' 5' end of the cDNA using a combination of gene specific and adaptor specific oligonucleotide primers. The PCR reaction is then repeated using 'nested' primers, that is, primers designed to anneal within the amplified product (typically an adaptor specific primer that anneals further 3' in the adaptor sequence and a gene specific primer that anneals further 5' in the known gene sequence). The products of this reaction can then be analysed by DNA sequencing and a full-length cDNA constructed either by joining the product directly to the existing cDNA to give a complete sequence, or carrying out a separate full-length PCR using the new sequence information for the design of the 5' primer.

Recombinant polypeptides of the present invention may be prepared by processes well known in the art from genetically engineered host cells comprising expression systems. Accordingly, in a further aspect, the present invention relates to an expression system which comprises a polynucleotide of the present invention, to host cells which are genetically engineered with such expression systems and to the production of polypeptides of the invention by recombinant techniques. Cell-free translation systems can also be employed to produce such proteins using RNAs derived from the DNA constructs of the present invention.

For recombinant production, host cells can be genetically engineered to incorporate expression systems or portions thereof for polynucleotides of the present invention. Introduction of polynucleotides into host cells can be effected by methods described in many standard laboratory manuals, such as Davis *et al.*, Basic Methods in Molecular Biology (1986) and Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989). Such methods include, for instance, calcium phosphate transfection, DEAE-dextran mediated transfection, transfection, microinjection, cationic lipid-mediated transfection, electroporation, transduction, scrape loading, ballistic introduction or infection.

The proteins of the invention may be coexpressed with thioredoxin in trans (TIT). Coexpression of thioredoxin in trans versus in cis keeps antigen free of thioredoxin without the need for protease. Thioredoxin coexpression eases the solubilisation of the proteins of the invention. Thioredoxin coexpression has also a significant impact on protein purification yield, on purified-protein solubility and quality.

Representative examples of appropriate hosts include bacterial cells, such as *Streptococci*, *Staphylococci*, *E. coli*, *Streptomyces* and *Bacillus subtilis* cells; fungal cells, such as yeast cells and *Aspergillus* cells; insect cells such as *Drosophila S2* and *Spodoptera Sf9* cells; animal cells such as CHO, COS, HeLa, C127, 3T3, BHK, HEK 293 and *Bowes* melanoma cells; and plant cells.

A great variety of expression systems can be used, for instance, chromosomal, episomal and virus-derived systems, e.g., vectors derived from bacterial plasmids, from bacteriophage, from transposons, from yeast episomes, from insertion elements, from yeast chromosomal elements, from viruses such as baculoviruses, papova viruses, such as SV40, vaccinia viruses, adenoviruses, fowl pox viruses, pseudorabies viruses and retroviruses, and vectors derived from combinations thereof, such as those derived from plasmid and bacteriophage genetic elements, such as cosmids and phagemids. The expression systems may contain control regions that regulate as well as engender expression. Generally, any system or vector which is able to maintain, propagate or express a polynucleotide to produce a polypeptide in a host may be used. The appropriate nucleotide sequence may be inserted into an expression system by any of a variety of well-known and routine techniques, such as, for example, those set forth in Sambrook *et al.*, *Molecular Cloning, A Laboratory Manual (supra)*. Appropriate secretion signals may be incorporated into the desired polypeptide to allow secretion of the translated protein into the lumen of the endoplasmic reticulum, the periplasmic space or the extracellular environment. These signals may be endogenous to the polypeptide or they may be heterologous signals.

The expression system may also be a recombinant live microorganism, such as a virus or bacterium. The gene of interest can be inserted into the genome of a live recombinant virus or bacterium. Inoculation and *in vivo* infection with this live vector will lead to *in vivo* expression of the antigen and induction of immune responses.

Therefore, in certain embodiments, polynucleotides encoding immunogenic polypeptides of the present invention are introduced into suitable mammalian host cells

for expression using any of a number of known viral-based systems. In one illustrative embodiment, retroviruses provide a convenient and effective platform for gene delivery systems. A selected nucleotide sequence encoding a polypeptide of the present invention can be inserted into a vector and packaged in retroviral particles using techniques known  
 5 in the art. The recombinant virus can then be isolated and delivered to a subject. A number of illustrative retroviral systems have been described (e.g., U.S. Pat. No. 5,219,740; Miller and Rosman (1989) *BioTechniques* 7:980-990; Miller, A. D. (1990) *Human Gene Therapy* 1:5-14; Scarpa *et al.* (1991) *Virology* 180:849-852; Burns *et al.* (1993) *Proc. Natl. Acad. Sci. USA* 90:8033-8037; and Boris-Lawrie and Temin (1993)  
 10 *Cur. Opin. Genet. Develop.* 3:102-109.

In addition, a number of illustrative adenovirus-based systems have also been described. Unlike retroviruses which integrate into the host genome, adenoviruses persist extrachromosomally thus minimizing the risks associated with insertional mutagenesis (Haj-Ahmad and Graham (1986) *J. Virol.* 57:267-274; Bett *et al.* (1993) *J. Virol.*  
 15 67:5911-5921; Mittereder *et al.* (1994) *Human Gene Therapy* 5:717-729; Seth *et al.* (1994) *J. Virol.* 68:933-940; Barr *et al.* (1994) *Gene Therapy* 1:51-58; Berkner, K. L. (1988) *BioTechniques* 6:616-629; and Rich *et al.* (1993) *Human Gene Therapy* 4:461-476).

Various adeno-associated virus (AAV) vector systems have also been developed  
 20 for polynucleotide delivery. AAV vectors can be readily constructed using techniques well known in the art. See, e.g., U.S. Pat. Nos. 5,173,414 and 5,139,941; International Publication Nos. WO 92/01070 and WO 93/03769; Lebkowski *et al.* (1988) *Molec. Cell. Biol.* 8:3988-3996; Vincent *et al.* (1990) *Vaccines* 90 (Cold Spring Harbor Laboratory Press); Carter, B. J. (1992) *Current Opinion in Biotechnology* 3:533-539; Muzyczka, N.  
 25 (1992) *Current Topics in Microbiol. and Immunol.* 158:97-129; Kotin, R. M. (1994) *Human Gene Therapy* 5:793-801; Shelling and Smith (1994) *Gene Therapy* 1:165-169; and Zhou *et al.* (1994) *J. Exp. Med.* 179:1867-1875.

Additional viral vectors useful for delivering the nucleic acid molecules encoding polypeptides of the present invention by gene transfer include those derived from the pox  
 30 family of viruses, such as vaccinia virus and avian poxvirus. By way of example, vaccinia virus recombinants expressing the novel molecules can be constructed as follows. The DNA encoding a polypeptide is first inserted into an appropriate vector so that it is adjacent to a vaccinia promoter and flanking vaccinia DNA sequences, such as the

sequence encoding thymidine kinase (TK). This vector is then used to transfect cells which are simultaneously infected with vaccinia. Homologous recombination serves to insert the vaccinia promoter plus the gene encoding the polypeptide of interest into the viral genome. The resulting TK.sup.(-) recombinant can be selected by culturing the cells in the presence of 5-bromodeoxyuridine and picking viral plaques resistant thereto.

A vaccinia-based infection/transfection system can be conveniently used to provide for inducible, transient expression or coexpression of one or more polypeptides described herein in host cells of an organism. In this particular system, cells are first infected *in vitro* with a vaccinia virus recombinant that encodes the bacteriophage T7 RNA polymerase. This polymerase displays exquisite specificity in that it only transcribes templates bearing T7 promoters. Following infection, cells are transfected with the polynucleotide or polynucleotides of interest, driven by a T7 promoter. The polymerase expressed in the cytoplasm from the vaccinia virus recombinant transcribes the transfected DNA into RNA which is then translated into polypeptide by the host translational machinery. The method provides for high level, transient, cytoplasmic production of large quantities of RNA and its translation products. See, e.g., Elroy-Stein and Moss, Proc. Natl. Acad. Sci. USA (1990) 87:6743-6747; Fuerst *et al.* Proc. Natl. Acad. Sci. USA (1986) 83:8122-8126.

Alternatively, avipoxviruses, such as the fowlpox and canarypox viruses, can also be used to deliver the coding sequences of interest. Recombinant avipox viruses, expressing immunogens from mammalian pathogens, are known to confer protective immunity when administered to non-avian species. The use of an Avipox vector is particularly desirable in human and other mammalian species since members of the Avipox genus can only productively replicate in susceptible avian species and therefore are not infective in mammalian cells. Methods for producing recombinant Avipoxviruses are known in the art and employ genetic recombination, as described above with respect to the production of vaccinia viruses. See, e.g., WO 91/12882; WO 89/03429; and WO 92/03545.

Any of a number of alphavirus vectors can also be used for delivery of polynucleotide compositions of the present invention, such as those vectors described in U.S. Patent Nos. 5,843,723; 6,015,686; 6,008,035 and 6,015,694. Certain vectors based on Venezuelan Equine Encephalitis (VEE) can also be used, illustrative examples of which can be found in U.S. Patent Nos. 5,505,947 and 5,643,576.



Moreover, molecular conjugate vectors, such as the adenovirus chimeric vectors described in Michael *et al. J. Biol. Chem.* (1993) 268:6866-6869 and Wagner *et al. Proc. Natl. Acad. Sci. USA* (1992) 89:6099-6103, can also be used for gene delivery under the invention.

5 Additional illustrative information on these and other known viral-based delivery systems can be found, for example, in Fisher-Hoch *et al., Proc. Natl. Acad. Sci. USA* 86:317-321, 1989; Flexner *et al., Ann. N.Y. Acad. Sci.* 569:86-103, 1989; Flexner *et al., Vaccine* 8:17-21, 1990; U.S. Patent Nos. 4,603,112, 4,769,330, and 5,017,487; WO 89/01973; U.S. Patent No. 4,777,127; GB 2,200,651; EP 0,345,242; WO 91/02805; 10 Berkner, *Biotechniques* 6:616-627, 1988; Rosenfeld *et al., Science* 252:431-434, 1991; Kolls *et al., Proc. Natl. Acad. Sci. USA* 91:215-219, 1994; Kass-Eisler *et al., Proc. Natl. Acad. Sci. USA* 90:11498-11502, 1993; Guzman *et al., Circulation* 88:2838-2848, 1993; and Guzman *et al., Cir. Res.* 73:1202-1207, 1993.

The recombinant live microorganisms described above can be virulent, or 15 attenuated in various ways in order to obtain live vaccines. Such live vaccines also form part of the invention.

In certain embodiments, a polynucleotide may be integrated into the genome of a target cell. This integration may be in the specific location and orientation via homologous recombination (gene replacement) or it may be integrated in a random, non-specific location (gene augmentation). In yet further embodiments, the polynucleotide 20 may be stably maintained in the cell as a separate, episomal segment of DNA. Such polynucleotide segments or "episomes" encode sequences sufficient to permit maintenance and replication independent of or in synchronization with the host cell cycle. The manner in which the expression construct is delivered to a cell and where in the cell 25 the polynucleotide remains is dependent on the type of expression construct employed.

In another embodiment of the invention, a polynucleotide is administered/delivered as "naked" DNA, for example as described in Ulmer *et al., Science* 259:1745-1749, 1993 and reviewed by Cohen, *Science* 259:1691-1692, 1993. The uptake of naked DNA may be increased by coating the DNA onto biodegradable 30 beads, which are efficiently transported into the cells.

In still another embodiment, a composition of the present invention can be delivered via a particle bombardment approach, many of which have been described. In one illustrative example, gas-driven particle acceleration can be achieved with devices

such as those manufactured by Powderject Pharmaceuticals PLC (Oxford, UK) and Powderject Vaccines Inc. (Madison, WI), some examples of which are described in U.S. Patent Nos. 5,846,796; 6,010,478; 5,865,796; 5,584,807; and EP Patent No. 0500 799. This approach offers a needle-free delivery approach wherein a dry powder formulation  
5 of microscopic particles, such as polynucleotide or polypeptide particles, are accelerated to high speed within a helium gas jet generated by a hand held device, propelling the particles into a target tissue of interest.

In a related embodiment, other devices and methods that may be useful for gas-driven needle-less injection of compositions of the present invention include those  
10 provided by Bioject, Inc. (Portland, OR), some examples of which are described in U.S. Patent Nos. 4,790,824; 5,064,413; 5,312,335; 5,383,851; 5,399,163; 5,520,639 and 5,993,412.

Polypeptides of the present invention can be recovered and purified from recombinant cell cultures by well-known methods including ammonium sulfate or ethanol  
15 precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxylapatite chromatography and lectin chromatography. Ion metal affinity chromatography (IMAC) may be employed for purification. Well known techniques for refolding proteins may be employed to regenerate active conformation when the  
20 polypeptide is denatured during intracellular synthesis, isolation and or purification.

Another important aspect of the invention relates to a method for inducing , re-inforcing or modulating an immunological response in a mammal which comprises inoculating the mammal with a fragment or the entire polypeptide or polynucleotide of the invention, adequate to produce antibody and/or T cell immune response for  
25 immunoprophylaxis or for therapeutic treatment of cancer, more particularly colorectal cancer, and autoimmune disease and related conditions. Yet another aspect of the invention relates to a method of inducing, re-inforcing or modulating immunological response in a mammal which comprises, delivering a polypeptide of the present invention via a vector or cell directing expression of the polynucleotide and coding for the  
30 polypeptide *in vivo* in order to induce such an immunological response to produce immune responses for prophylaxis or treatment of said mammal from diseases.

A further aspect of the invention relates to an immunological/vaccine formulation (composition) and to their use in medicine. These compositions, when introduced into a

mammalian host, induce, re-inforce or modulate an immunological response in that mammal to a polypeptide of the present invention wherein the composition comprises a polypeptide or polynucleotide of the invention or an immunological fragment thereof as herein before defined. More particularly the immunogenic composition according to the present invention comprises a safe and effective amount of a CASB7439 polypeptide, or immunogenic fragment thereof wherein the CASB7439 polypeptide is selected from the group comprising SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:7, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12 or SEQ ID NO:14. In another embodiment, the immunogenic composition comprises a safe and effective amount of a CASB7439 -encoding polynucleotide, or fragment thereof wherein the CASB7439-encoding polynucleotide is selected from the group comprising SEQ ID NO:1, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:13 or SEQ ID NO:15.

The vaccine formulation according to the invention may further comprise a suitable, i.e. pharmaceutically acceptable carrier. Since a polypeptide may be broken down in the stomach, it may be administered parenterally (for instance, subcutaneous, intramuscular, intravenous, or intradermal injection). Formulations suitable for parenteral administration include aqueous and non-aqueous sterile injection solutions which may contain anti-oxidants, buffers, bacteriostats and solutes which render the formulation isotonic with the blood of the recipient; and aqueous and non-aqueous sterile suspensions which may include suspending agents or thickening agents. The formulations may be presented in unit-dose or multi-dose containers, for example, sealed ampoules and vials and may be stored in a freeze-dried condition requiring only the addition of the sterile liquid carrier immediately prior to use.

A further aspect of the invention relates to the *in vitro* induction of immune responses to a fragment or the entire polypeptide or polynucleotide of the present invention or a molecule comprising the polypeptide or polynucleotide of the present invention, using cells from the immune system of a mammal, and reinfusing these activated immune cells of the mammal for the treatment of disease. Activation of the cells from the immune system is achieved by *in vitro* incubation with the entire polypeptide or polynucleotide of the present invention or a molecule comprising the polypeptide or polynucleotide of the present invention in the presence or absence of various immunomodulator molecules.

A further aspect of the invention relates to the immunization of a mammal by administration of antigen presenting cells modified by *in vitro* loading with part or the entire polypeptide of the present invention or a molecule comprising the polypeptide of the present invention and administered *in vivo* in an immunogenic way. Alternatively, antigen presenting cells can be transfected *in vitro* with a vector containing a fragment or the entire polynucleotide of the present invention or a molecule comprising the polynucleotide of the present invention, such as to express the corresponding polypeptide, and administered *in vivo* in an immunogenic way. Accordingly, the pharmaceutical compositions of the invention will comprise an effective amount of antigen presenting cells, modified by *in vitro* loading with a CASB7439 polypeptide, or genetically modified *in vitro* to express a CASB7439 polypeptide and a pharmaceutically effective carrier.

According to another embodiment, the pharmaceutical/immunogenic compositions described herein will comprise one or more immunostimulants in addition to the immunogenic polynucleotide, polypeptide, antibody, T-cell and/or antigen presenting cell (APC) compositions of this invention. Accordingly there is herein provided a process for the production of said immunogenic composition, comprising admixing a CASB7439 polypeptide or a CASB7439 -encoding polynucleotide with a suitable adjuvant/immunostimulant, diluent or other pharmaceutically acceptable carrier. An immunostimulant refers to essentially any substance that enhances or potentiates an immune response (antibody and/or cell-mediated) to an exogenous antigen. One type of immunostimulant comprises an adjuvant. Many adjuvants contain a substance designed to protect the antigen from rapid catabolism, such as aluminum hydroxide or mineral oil, and a stimulator of immune responses, such as lipid A, *Bordetella pertussis* or Mycobacterium tuberculosis derived proteins. Certain adjuvants are commercially available as, for example, Freund's Incomplete Adjuvant and Complete Adjuvant (Difco Laboratories, Detroit, MI); Merck Adjuvant 65 (Merck and Company, Inc., Rahway, NJ); AS-2 (SmithKline Beecham, Philadelphia, PA); aluminum salts such as aluminum hydroxide gel (alum) or aluminum phosphate; salts of calcium, iron or zinc; an insoluble suspension of acylated tyrosine; acylated sugars; cationically or anionically derivatized polysaccharides; polyphosphazenes; biodegradable microspheres; monophosphoryl lipid A and quil A. Cytokines, such as GM-CSF, interleukin-2, -7, -12, and other like growth factors, may also be used as adjuvants.

Within certain embodiments of the invention, the adjuvant composition may be one that induces an immune response predominantly of the Th1 type. High levels of Th1-type cytokines (e.g., IFN- $\gamma$ , TNF $\alpha$ , IL-2 and IL-12) tend to favor the induction of cell mediated immune responses to an administered antigen. In contrast, high levels of Th2-type cytokines (e.g., IL-4, IL-5, IL-6 and IL-10) tend to favor the induction of humoral immune responses. Following application of a vaccine as provided herein, a patient will support an immune response that includes Th1- and Th2-type responses. Within an embodiment, in which a response is predominantly Th1-type, the level of Th1-type cytokines will increase to a greater extent than the level of Th2-type cytokines. The levels of these cytokines may be readily assessed using standard assays. For a review of the families of cytokines, see Mosmann and Coffman, *Ann. Rev. Immunol.* 7:145-173, 1989.

Certain adjuvants for eliciting a predominantly Th1-type response include, for example, a combination of monophosphoryl lipid A, including 3-de-O-acylated monophosphoryl lipid A, together with an aluminum salt. MPL<sup>®</sup> adjuvants are available from Corixa Corporation (Seattle, WA; *see*, for example, US Patent Nos. 4,436,727; 4,877,611; 4,866,034 and 4,912,094). CpG-containing oligonucleotides (in which the CpG dinucleotide is unmethylated) also induce a predominantly Th1 response. Such oligonucleotides are well known and are described, for example, in WO 96/02555, WO 99/33488 and U.S. Patent Nos. 6,008,200 and 5,856,462. Immunostimulatory DNA sequences are also described, for example, by Sato *et al.*, *Science* 273:352, 1996. Another adjuvant comprises a saponin, such as Quil A, or derivatives thereof, including QS21 and QS7 (Aquila Biopharmaceuticals Inc., Framingham, MA); Escin; Digitonin; or *Gypsophila* or *Chenopodium quinoa* saponins. Other formulations include more than one saponin in the adjuvant combinations of the present invention, for example combinations of at least two of the following group comprising QS21, QS7, Quil A,  $\beta$ -escin, or digitonin.

Alternatively the saponin formulations may be combined with vaccine vehicles composed of chitosan or other polycationic polymers, polylactide and polylactide-co-glycolide particles, poly-N-acetyl glucosamine-based polymer matrix, particles composed of polysaccharides or chemically modified polysaccharides, liposomes and lipid-based particles, particles composed of glycerol monoesters, etc. The saponins may also be formulated in the presence of cholesterol to form particulate structures such as liposomes

or ISCOMs. Furthermore, the saponins may be formulated together with a polyoxyethylene ether or ester, in either a non-particulate solution or suspension, or in a particulate structure such as a paucilamellar liposome or ISCOM. The saponins may also be formulated with excipients such as Carbopol<sup>R</sup> to increase viscosity, or may be  
 5 formulated in a dry powder form with a powder excipient such as lactose.

In one embodiment, the adjuvant system includes the combination of a monophosphoryl lipid A and a saponin derivative, such as the combination of QS21 and 3D-MPL<sup>®</sup> adjuvant, as described in WO 94/00153, or a less reactogenic composition where the QS21 is quenched with cholesterol, as described in WO 96/33739. Other  
 10 formulations comprise an oil-in-water emulsion and tocopherol. Another adjuvant formulation employing QS21, 3D-MPL<sup>®</sup> adjuvant and tocopherol in an oil-in-water emulsion is described in WO 95/17210.

Another enhanced adjuvant system involves the combination of a CpG-containing oligonucleotide and a saponin derivative particularly the combination of CpG and QS21  
 15 as disclosed in WO 00/09159. The formulation may additionally comprise an oil in water emulsion and tocopherol.

Additional illustrative adjuvants for use in the pharmaceutical compositions of the invention include Montanide ISA 720 (Seppic, France), SAF (Chiron, California, United States), ISCOMS (CSL), MF-59 (Chiron), the SBAS series of adjuvants (e.g., SBAS-2 or  
 20 SBAS-4, available from SmithKline Beecham, Rixensart, Belgium), Detox (Enhanzyn<sup>®</sup>) (Corixa, Hamilton, MT), RC-529 (Corixa, Hamilton, MT) and other aminoalkyl glucosaminide 4-phosphates (AGPs), such as those described in pending U.S. Patent Application Serial Nos. 08/853,826 and 09/074,720, the disclosures of which are incorporated herein by reference in their entireties, and polyoxyethylene ether adjuvants  
 25 such as those described in WO 99/52549A1.

Other adjuvants include adjuvant molecules of the general formula (I):



Wherein, n is 1-50, A is a bond or -C(O)-, R is C<sub>1-50</sub> alkyl or Phenyl C<sub>1-50</sub> alkyl.

One embodiment of the present invention consists of a vaccine formulation comprising a  
 30 polyoxyethylene ether of general formula (I), wherein n is between 1 and 50, or 4-24, or

9; the R component is C1-50, or C4-C20 alkyl or C12 alkyl, and A is a bond. The concentration of the polyoxyethylene ethers should be in the range 0.1-20%, or from 0.1-10%, or in the range 0.1-1%. Polyoxyethylene ethers may be selected from the following group: polyoxyethylene-9-lauryl ether, polyoxyethylene-9-stearyl ether,  
 5 polyoxyethylene-8-stearyl ether, polyoxyethylene-4-lauryl ether, polyoxyethylene-35-lauryl ether, and polyoxyethylene-23-lauryl ether. Polyoxyethylene ethers such as polyoxyethylene lauryl ether are described in the Merck index (12th edition: entry 7717). These adjuvant molecules are described in WO 99/52549.

10 The polyoxyethylene ether according to the general formula (I) above may, if desired, be combined with another adjuvant. For example, an adjuvant combination may be with CpG as described in the pending UK patent application GB 9820956.2.

A carrier is also present in the vaccine composition according to the invention. The carrier may be an oil in water emulsion, or an aluminium salt, such as aluminium phosphate or aluminium hydroxide.

15 A oil-in-water emulsion may comprise a metabolisable oil, such as squalene, alpha tocopherol and Tween 80. In one aspect, the antigens in the vaccine composition according to the invention are combined with QS21 and 3D-MPL in such an emulsion. Additionally the oil in water emulsion may contain span 85 and/or lecithin and/or tricaprillin.

20 Typically for human administration QS21 and 3D-MPL will be present in a vaccine in the range of 1µg - 200µg, such as 10-100µg, or 10µg - 50µg per dose. Typically the oil in water will comprise from 2 to 10% squalene, from 2 to 10% alpha tocopherol and from 0.3 to 3% tween 80. The ratio of squalene may be: alpha tocopherol is equal to or less than 1 as this provides a more stable emulsion. Span 85 may also be  
 25 present at a level of 1%. In some cases it may be advantageous that the vaccines of the present invention will further contain a stabiliser.

Non-toxic oil in water emulsions may contain a non-toxic oil, e.g. squalene or squalene, an emulsifier, e.g. Tween 80, in an aqueous carrier. The aqueous carrier may be, for example, phosphate buffered saline.

30 A particularly potent adjuvant formulation involving QS21, 3D-MPL and tocopherol in an oil in water emulsion is described in WO 95/17210.

The present invention also provides a polyvalent vaccine composition comprising a vaccine formulation of the invention in combination with other antigens, in particular antigens useful for treating cancers, more particularly colorectal cancer, autoimmune diseases and related conditions. Such a polyvalent vaccine composition may include a  
 5 TH-1 inducing adjuvant as hereinbefore described.

According to another embodiment of this invention, an immunogenic composition described herein is delivered to a host via antigen presenting cells (APCs), such as dendritic cells, macrophages, B cells, monocytes and other cells that may be engineered to be efficient APCs. Such cells may, but need not, be genetically modified to increase  
 10 the capacity for presenting the antigen, to improve activation and/or maintenance of the T cell response, to have anti-tumor effects *per se* and/or to be immunologically compatible with the receiver (i.e., matched HLA haplotype). APCs may generally be isolated from any of a variety of biological fluids and organs, including tumor and peritumoral tissues, and may be autologous, allogeneic, syngeneic or xenogeneic cells.

Certain embodiments of the present invention may use dendritic cells or progenitors thereof as antigen-presenting cells. Dendritic cells are highly potent APCs (Banchereau and Steinman, Nature 392:245-251, 1998) and have been shown to be effective as a physiological adjuvant for eliciting prophylactic or therapeutic antitumor immunity (see Timmerman and Levy, Ann. Rev. Med. 50:507-529, 1999). In general,  
 20 dendritic cells may be identified based on their typical shape (stellate in situ, with marked cytoplasmic processes (dendrites) visible *in vitro*), their ability to take up, process and present antigens with high efficiency and their ability to activate naïve T cell responses. Dendritic cells may, of course, be engineered to express specific cell-surface receptors or ligands that are not commonly found on dendritic cells *in vivo* or ex vivo, and such  
 25 modified dendritic cells are contemplated by the present invention. As an alternative to dendritic cells, secreted vesicles antigen-loaded dendritic cells (called exosomes) may be used within a vaccine (see Zitvogel *et al.*, Nature Med. 4:594-600, 1998).

Dendritic cells and progenitors may be obtained from peripheral blood, bone marrow, tumor-infiltrating cells, peritumoral tissues-infiltrating cells, lymph nodes,  
 30 spleen, skin, umbilical cord blood or any other suitable tissue or fluid. For example, dendritic cells may be differentiated ex vivo by adding a combination of cytokines such as GM-CSF, IL-4, IL-13 and/or TNF $\alpha$  to cultures of monocytes harvested from peripheral blood. Alternatively, CD34 positive cells harvested from peripheral blood,



umbilical cord blood or bone marrow may be differentiated into dendritic cells by adding to the culture medium combinations of GM-CSF, IL-3, TNF $\alpha$ , CD40 ligand, LPS, flt3 ligand and/or other compound(s) that induce differentiation, maturation and proliferation of dendritic cells.

5 Dendritic cells are conveniently categorized as "immature" and "mature" cells, which allows a simple way to discriminate between two well characterised phenotypes. However, this nomenclature should not be construed to exclude all possible intermediate stages of differentiation. Immature dendritic cells are characterised as APC with a high capacity for antigen uptake and processing, which correlates with the high expression of  
10 Fc $\gamma$  receptor and mannose receptor. The mature phenotype is typically characterized by a lower expression of these markers, but a high expression of cell surface molecules responsible for T cell activation such as class I and class II MHC, adhesion molecules (e.g., CD54 and CD11) and costimulatory molecules (e.g., CD40, CD80, CD86 and 4-1BB).

15 APCs may generally be transfected with a polynucleotide of the invention (or portion or other variant thereof) such that the encoded polypeptide, or an immunogenic portion thereof, is expressed on the cell surface. Such transfection may take place *ex vivo*, and a pharmaceutical composition comprising such transfected cells may then be used for therapeutic purposes, as described herein. Alternatively, a gene delivery vehicle  
20 that targets a dendritic or other antigen presenting cell may be administered to a patient, resulting in transfection that occurs *in vivo*. *In vivo* and *ex vivo* transfection of dendritic cells, for example, may generally be performed using any methods known in the art, such as those described in WO 97/24447, or the gene gun approach described by Mahvi *et al.*, Immunology and cell Biology 75:456-460, 1997. Antigen loading of dendritic cells may  
25 be achieved by incubating dendritic cells or progenitor cells with the tumor polypeptide, DNA (naked or within a plasmid vector) or RNA; or with antigen-expressing recombinant bacterium or viruses (e.g., vaccinia, fowlpox, adenovirus or lentivirus vectors). Prior to loading, the polypeptide may be covalently conjugated to an immunological partner that provides T cell help (e.g., a carrier molecule). Alternatively,  
30 a dendritic cell may be pulsed with a non-conjugated immunological partner, separately or in the presence of the polypeptide.

While any suitable carrier known to those of ordinary skill in the art may be employed in the pharmaceutical compositions of this invention, the type of carrier will

typically vary depending on the mode of administration. Compositions of the present invention may be formulated for any appropriate manner of administration, including for example, topical, oral, nasal, mucosal, intravenous, intracranial, intraperitoneal, subcutaneous and intramuscular administration.

5 Carriers for use within such pharmaceutical compositions are biocompatible, and may also be biodegradable. In certain embodiments, the formulation may provide a relatively constant level of active component release. In other embodiments, however, a more rapid rate of release immediately upon administration may be desired. The formulation of such compositions is well within the level of ordinary skill in the art using  
10 known techniques. Illustrative carriers useful in this regard include microparticles of poly(lactide-co-glycolide), polyacrylate, latex, starch, cellulose, dextran and the like. Other illustrative delayed-release carriers include supramolecular biovectors, which comprise a non-liquid hydrophilic core (e.g., a cross-linked polysaccharide or oligosaccharide) and, optionally, an external layer comprising an amphiphilic compound,  
15 such as a phospholipid (see e.g., U.S. Patent No. 5,151,254 and PCT applications WO 94/20078, WO/94/23701 and WO 96/06638). The amount of active compound contained within a sustained release formulation depends upon the site of implantation, the rate and expected duration of release and the nature of the condition to be treated or prevented.

In another illustrative embodiment, biodegradable microspheres (e.g., polylactate  
20 polyglycolate) are employed as carriers for the compositions of this invention. Suitable biodegradable microspheres are disclosed, for example, in U.S. Patent Nos. 4,897,268; 5,075,109; 5,928,647; 5,811,128; 5,820,883; 5,853,763; 5,814,344, 5,407,609 and 5,942,252. Modified hepatitis B core protein carrier systems, such as described in WO/99 40934, and references cited therein, will also be useful for many applications.  
25 Another illustrative carrier/delivery system employs a carrier comprising particulate-protein complexes, such as those described in U.S. Patent No. 5,928,647, which are capable of inducing a class I-restricted cytotoxic T lymphocyte responses in a host.

The pharmaceutical compositions of the invention will often further comprise one or more buffers (e.g., neutral buffered saline or phosphate buffered saline), carbohydrates  
30 (e.g., glucose, mannose, sucrose or dextrans), mannitol, proteins, polypeptides or amino acids such as glycine, antioxidants, bacteriostats, chelating agents such as EDTA or glutathione, adjuvants (e.g., aluminum hydroxide), solutes that render the formulation isotonic, hypotonic or weakly hypertonic with the blood of a recipient, suspending agents,

thickening agents and/or preservatives. Alternatively, compositions of the present invention may be formulated as a lyophilizate.

The pharmaceutical compositions described herein may be presented in unit-dose or multi-dose containers, such as sealed ampoules or vials. Such containers are typically sealed in such a way to preserve the sterility and stability of the formulation until use. In general, formulations may be stored as suspensions, solutions or emulsions in oily or aqueous vehicles. Alternatively, a pharmaceutical composition may be stored in a freeze-dried condition requiring only the addition of a sterile liquid carrier immediately prior to use.

The development of suitable dosing and treatment regimens for using the particular compositions described herein in a variety of treatment regimens, including e.g., oral, parenteral, intravenous, intranasal, and intramuscular administration and formulation, is well known in the art, some of which are briefly discussed below for general purposes of illustration.

In certain applications, the pharmaceutical compositions disclosed herein may be delivered via oral administration to an animal. As such, these compositions may be formulated with an inert diluent or with an assimilable edible carrier, or they may be enclosed in hard- or soft-shell gelatin capsule, or they may be compressed into tablets, or they may be incorporated directly with the food of the diet.

The active compounds may even be incorporated with excipients and used in the form of ingestible tablets, buccal tables, troches, capsules, elixirs, suspensions, syrups, wafers, and the like (see, for example, Mathiowitz *et al.*, Nature 1997 Mar 27;386(6623):410-4; Hwang *et al.*, Crit Rev Ther Drug Carrier Syst 1998;15(3):243-84; U. S. Patent 5,641,515; U. S. Patent 5,580,579 and U. S. Patent 5,792,451). Tablets, troches, pills, capsules and the like may also contain any of a variety of additional components, for example, a binder, such as gum tragacanth, acacia, cornstarch, or gelatin; excipients, such as dicalcium phosphate; a disintegrating agent, such as corn starch, potato starch, alginic acid and the like; a lubricant, such as magnesium stearate; and a sweetening agent, such as sucrose, lactose or saccharin may be added or a flavoring agent, such as peppermint, oil of wintergreen, or cherry flavoring. When the dosage unit form is a capsule, it may contain, in addition to materials of the above type, a liquid carrier. Various other materials may be present as coatings or to otherwise modify the physical form of the dosage unit. For instance, tablets, pills, or capsules may be coated

with shellac, sugar, or both. Of course, any material used in preparing any dosage unit form should be pharmaceutically pure and substantially non-toxic in the amounts employed. In addition, the active compounds may be incorporated into sustained-release preparation and formulations.

5           Typically, these formulations will contain at least about 0.1% of the active compound or more, although the percentage of the active ingredient(s) may, of course, be varied and may conveniently be between about 1 or 2% and about 60% or 70% or more of the weight or volume of the total formulation. Naturally, the amount of active compound(s) in each therapeutically useful composition may be prepared is such a way  
10       that a suitable dosage will be obtained in any given unit dose of the compound. Factors such as solubility, bioavailability, biological half-life, route of administration, product shelf life, as well as other pharmacological considerations will be contemplated by one skilled in the art of preparing such pharmaceutical formulations, and as such, a variety of dosages and treatment regimens may be desirable.

15           For oral administration the compositions of the present invention may alternatively be incorporated with one or more excipients in the form of a mouthwash, dentifrice, buccal tablet, oral spray, or sublingual orally-administered formulation. Alternatively, the active ingredient may be incorporated into an oral solution such as one containing sodium borate, glycerin and potassium bicarbonate, or dispersed in a  
20       dentifrice, or added in a therapeutically-effective amount to a composition that may include water, binders, abrasives, flavoring agents, foaming agents, and humectants. Alternatively the compositions may be fashioned into a tablet or solution form that may be placed under the tongue or otherwise dissolved in the mouth.

          In certain circumstances it will be desirable to deliver the pharmaceutical  
25       compositions disclosed herein parenterally, intravenously, intramuscularly, or even intraperitoneally. Such approaches are well known to the skilled artisan, some of which are further described, for example, in U. S. Patent 5,543,158; U. S. Patent 5,641,515 and U. S. Patent 5,399,363. In certain embodiments, solutions of the active compounds as free base or pharmacologically acceptable salts may be prepared in water suitably mixed  
30       with a surfactant, such as hydroxypropylcellulose. Dispersions may also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary

conditions of storage and use, these preparations generally will contain a preservative to prevent the growth of microorganisms.

Illustrative pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions (for example, see U. S. Patent 5,466,468). In all cases the form must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms, such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (e.g., glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and/or vegetable oils. Proper fluidity may be maintained, for example, by the use of a coating, such as lecithin, by the maintenance of the required particle size in the case of dispersion and/or by the use of surfactants. The prevention of the action of microorganisms can be facilitated by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, isotonic agents may be included, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

In one embodiment, for parenteral administration in an aqueous solution, the solution should be suitably buffered if necessary and the liquid diluent first rendered isotonic with sufficient saline or glucose. These particular aqueous solutions are especially suitable for intravenous, intramuscular, subcutaneous and intraperitoneal administration. In this connection, a sterile aqueous medium that can be employed will be known to those of skill in the art in light of the present disclosure. For example, one dosage may be dissolved in 1 ml of isotonic NaCl solution and either added to 1000 ml of hypodermoclysis fluid or injected at the proposed site of infusion, (see for example, "Remington's Pharmaceutical Sciences" 15th Edition, pages 1035-1038 and 1570-1580). Some variation in dosage will necessarily occur depending on the condition of the subject being treated. Moreover, for human administration, preparations will meet sterility, pyrogenicity, and the general safety and purity standards as required by FDA Office of Biologics standards.

In another embodiment of the invention, the compositions disclosed herein may be formulated in a neutral or salt form. Illustrative pharmaceutically-acceptable salts include the acid addition salts (formed with the free amino groups of the protein) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, histidine, procaine and the like. Upon formulation, solutions will be administered in a manner compatible with the dosage formulation and in such amount as is therapeutically effective.

The carriers can further comprise any and all solvents, dispersion media, vehicles, coatings, diluents, antibacterial and antifungal agents, isotonic and absorption delaying agents, buffers, carrier solutions, suspensions, colloids, and the like. The use of such media and agents for pharmaceutical active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredient, its use in the therapeutic compositions is contemplated. Supplementary active ingredients can also be incorporated into the compositions. The phrase "pharmaceutically-acceptable" refers to molecular entities and compositions that do not produce an allergic or similar untoward reaction when administered to a human.

In certain embodiments, the pharmaceutical compositions may be delivered by intranasal sprays, inhalation, and/or other aerosol delivery vehicles. Methods for delivering genes, nucleic acids, and peptide compositions directly to the lungs via nasal aerosol sprays has been described, e.g., in U. S. Patent 5,756,353 and U. S. Patent 5,804,212. Likewise, the delivery of drugs using intranasal microparticle resins (Takenaga *et al.*, J Controlled Release 1998 Mar 2;52(1-2):81-7) and lysophosphatidyl-glycerol compounds (U. S. Patent 5,725,871) are also well-known in the pharmaceutical arts. Likewise, illustrative transmucosal drug delivery in the form of a polytetrafluoroethylene support matrix is described in U. S. Patent 5,780,045.

In certain embodiments, liposomes, nanocapsules, microparticles, lipid particles, vesicles, and the like, are used for the introduction of the compositions of the present invention into suitable host cells/organisms. In particular, the compositions of the present invention may be formulated for delivery either encapsulated in a lipid particle, a liposome, a vesicle, a nanosphere, or a nanoparticle or the like. Alternatively,

compositions of the present invention can be bound, either covalently or non-covalently, to the surface of such carrier vehicles.

The formation and use of liposome and liposome-like preparations as potential drug carriers is generally known to those of skill in the art (see for example, Lasic, Trends  
 5 Biotechnol 1998 Jul;16(7):307-21; Takakura, Nippon Rinsho 1998 Mar;56(3):691-5; Chandran *et al.*, Indian J Exp Biol. 1997 Aug;35(8):801-9; Margalit, Crit Rev Ther Drug Carrier Syst. 1995;12(2-3):233-61; U.S. Patent 5,567,434; U.S. Patent 5,552,157; U.S. Patent 5,565,213; U.S. Patent 5,738,868 and U.S. Patent 5,795,587, each specifically incorporated herein by reference in its entirety).

10 Liposomes have been used successfully with a number of cell types that are normally difficult to transfect by other procedures, including T cell suspensions, primary hepatocyte cultures and PC 12 cells (Renneisen *et al.*, J Biol Chem. 1990 Sep 25;265(27):16337-42; Muller *et al.*, DNA Cell Biol. 1990 Apr;9(3):221-9). In addition, liposomes are free of the DNA length constraints that are typical of viral-based delivery  
 15 systems. Liposomes have been used effectively to introduce genes, various drugs, radiotherapeutic agents, enzymes, viruses, transcription factors, allosteric effectors and the like, into a variety of cultured cell lines and animals. Furthermore, the use of liposomes does not appear to be associated with autoimmune responses or unacceptable toxicity after systemic delivery.

20 In certain embodiments, liposomes are formed from phospholipids that are dispersed in an aqueous medium and spontaneously form multilamellar concentric bilayer vesicles (also termed multilamellar vesicles (MLVs)).

Alternatively, in other embodiments, the invention provides for pharmaceutically-acceptable nanocapsule formulations of the compositions of the present invention.

25 Nanocapsules can generally entrap compounds in a stable and reproducible way (see, for example, Quintanar-Guerrero *et al.*, Drug Dev Ind Pharm. 1998 Dec;24(12):1113-28). To avoid side effects due to intracellular polymeric overloading, such ultrafine particles (sized around 0.1  $\mu\text{m}$ ) may be designed using polymers able to be degraded *in vivo*. Such particles can be made as described, for example, by Couvreur *et al.*, Crit Rev Ther Drug  
 30 Carrier Syst. 1988;5(1):1-20; zur Muhlen *et al.*, Eur J Pharm Biopharm. 1998 Mar;45(2):149-55; Zambaux *et al.* J Controlled Release. 1998 Jan 2;50(1-3):31-40; and U. S. Patent 5,145,684.

This invention also relates to the use of polynucleotides, in the form of primers derived from the polynucleotides of the present invention, and of polypeptides, in the form of antibodies or reagents specific for the polypeptide of the present invention, as diagnostic reagents.

5           The identification of genetic or biochemical markers in blood or tissues that will enable the detection of very early changes along the carcinogenesis pathway will help in determining the best treatment for the patient. Surrogate tumour markers, such as polynucleotide expression, can be used to diagnose different forms and states of cancer. The identification of expression levels of the polynucleotides of the invention will be  
10       useful in both the staging of the cancerous disorder and grading the nature of the cancerous tissue. The staging process monitors the advancement of the cancer and is determined on the presence or absence of malignant tissue in the areas biopsied. The polynucleotides of the invention can help to perfect the staging process by identifying markers for the aggressivity of a cancer, for example the presence in different areas of the  
15       body. The grading of the cancer describes how closely a tumour resembles normal tissue of its same type and is assessed by its cell morphology and other markers of differentiation. The polynucleotides of the invention can be useful in determining the tumour grade as they can help in the determination of the differentiation status of the cells of a tumour.

20           The diagnostic assays offer a process for diagnosing or determining a susceptibility to cancers, autoimmune disease and related conditions through diagnosis by methods comprising determining from a sample derived from a subject an abnormally decreased or increased level of polypeptide or mRNA. This method of diagnosis is known as differential expression. The expression of a particular gene is compared  
25       between a diseased tissue and a normal tissue. A difference between the polynucleotide-related gene, mRNA, or protein in the two tissues is compared, for example in molecular weight, amino acid or nucleotide sequence, or relative abundance, indicates a change in the gene, or a gene which regulates it, in the tissue of the human that was suspected of being diseased.

30           Decreased or increased expression can be measured at the RNA level. PolyA RNA is first isolated from the two tissues and the detection of mRNA encoded by a gene corresponding to a differentially expressed polynucleotide of the invention can be detected by, for example, in situ hybridization in tissue sections, reverse transcriptase-



PCR, using Northern blots containing poly A+ mRNA, or any other direct or indirect RNA detection method. An increased or decreased expression of a given RNA in a diseased tissue compared to a normal tissue suggests that the transcript and/or the expressed protein has a role in the disease. Thus detection of a higher or lower level of mRNA corresponding to SEQ ID NO: 1 relative to normal level is indicative of the presence of cancer in the patient.

mRNA expression levels in a sample can be determined by generation of a library of expressed sequence tags (ESTs) from the sample. The relative representation of ESTs in the library can be used to assess the relative representation of the gene transcript in the starting sample. The EST analysis of the test can then be compared to the EST analysis of a reference sample to determine the relative expression levels of the polynucleotide of interest.

Other mRNA analyses can be carried out using serial analysis of gene expression (SAGE) methodology (Velculescu et. Al. Science (1995) 270:484) , differential display methodology (For example, US 5,776,683) or hybridization analysis which relies on the specificity of nucleotide interactions.

Alternatively, the comparison could be made at the protein level. The protein sizes in the two tissues may be compared using antibodies to detect polypeptides in Western blots of protein extracts from the two tissues. Expression levels and subcellular localization may also be detected immunologically using antibodies to the corresponding protein. Further assay techniques that can be used to determine levels of a protein, such as a polypeptide of the present invention, in a sample derived from a host are well-known to those of skill in the art. A raised or decreased level of polypeptide expression in the diseased tissue compared with the same protein expression level in the normal tissue indicates that the expressed protein may be involved in the disease.

In the assays of the present invention, the diagnosis can be determined by detection of gene product expression levels encoded by at least one sequence set forth in SEQ ID NO: 1. A comparison of the mRNA or protein levels in a diseased versus normal tissue may also be used to follow the progression or remission of a disease.

A large number of polynucleotide sequences in a sample can be assayed using polynucleotide arrays. These can be used to examine differential expression of genes and to determine gene function. For example, arrays of the polynucleotide sequences SEQ ID NO:1 can be used to determine if any of the polynucleotides are differentially expressed

between a normal and cancer cell. In one embodiment of the invention, an array of oligonucleotides probes comprising the SEQ ID NO:1 nucleotide sequence or fragments thereof can be constructed to conduct efficient screening of e.g., genetic mutations.

Array technology methods are well known and have general applicability and can be used to address a variety of questions in molecular genetics including gene expression, genetic linkage, and genetic variability (see for example: M.Chee *et al.*, Science, Vol 274, pp 610-613 (1996)).

“Diagnosis” as used herein includes determination of a subject’s susceptibility to a disease, determination as to whether a subject presently has the disease, and also the prognosis of a subject affected by the disease.

The present invention, further relates to a diagnostic kit for performing a diagnostic assay which comprises:

- (a) a polynucleotide of the present invention, which may be the nucleotide sequence of SEQ ID NO: 1, or a fragment thereof;
- (b) a nucleotide sequence complementary to that of (a), which may be the nucleotide sequence of SEQ ID NO: 6;
- (c) a polypeptide of the present invention, which may be the polypeptide of SEQ ID NO: 2 or 3, or a fragment thereof; or
- (d) an antibody to a polypeptide of the present invention, which may be the polypeptide of SEQ ID NO:2 or 3.

The nucleotide sequences of the present invention are also valuable for chromosomal localisation. The sequence is specifically targeted to, and can hybridize with, a particular location on an individual human chromosome. The mapping of relevant sequences to chromosomes according to the present invention is an important first step in correlating those sequences with gene associated disease. Once a sequence has been mapped to a precise chromosomal location, the physical position of the sequence on the chromosome can be correlated with genetic map data. Such data are found in, for example, V. McKusick, Mendelian Inheritance in Man (available on-line through Johns Hopkins University Welch Medical Library). The relationship between genes and diseases that have been mapped to the same chromosomal region are then identified through linkage analysis (coinheritance of physically adjacent genes). The differences in the cDNA or genomic sequence between affected and unaffected individuals can also be determined.

The polypeptides of the invention or their fragments or analogs thereof, or cells expressing them, can also be used as immunogens to produce antibodies immunospecific for polypeptides of the present invention. The term "immunospecific" means that the antibodies have substantially greater affinity for the polypeptides of the invention than  
5 their affinity for other related polypeptides in the prior art.

In a further aspect the invention provides an antibody immunospecific for a polypeptide according to the invention or an immunological fragment thereof as hereinbefore defined. The antibody may be a monoclonal antibody.

Antibodies generated against polypeptides of the present invention may be  
10 obtained by administering the polypeptides or epitope-bearing fragments, analogs or cells to an animal, which may be a non-human animal, using routine protocols. For preparation of monoclonal antibodies, any technique which provides antibodies produced by continuous cell line cultures can be used. Examples include the hybridoma technique (Kohler, G. and Milstein, C., *Nature* (1975) 256:495-497), the trioma technique, the  
15 human B-cell hybridoma technique (Kozbor *et al.*, *Immunology Today* (1983) 4:72) and the EBV-hybridoma technique (Cole *et al.*, *Monoclonal Antibodies and Cancer Therapy*, 77-96, Alan R. Liss, Inc., 1985).

Techniques for the production of single chain antibodies, such as those described in U.S. Patent No. 4,946,778, can also be adapted to produce single chain antibodies to  
20 polypeptides of this invention. Also, transgenic mice, or other organisms, including other mammals, may be used to express humanized antibodies.

The above-described antibodies may be employed to isolate or to identify clones expressing the polypeptide or to purify the polypeptides by affinity chromatography. The antibody of the invention may also be employed to prevent or treat cancer,  
25 particularly colorectal cancer, autoimmune disease and related conditions.

Another aspect of the invention relates to a method for inducing or modulating an immunological response in a mammal which comprises inoculating the mammal with a polypeptide of the present invention, adequate to produce antibody and/or T cell immune response to protect or ameliorate the symptoms or progression of the disease. Yet  
30 another aspect of the invention relates to a method of inducing or modulating immunological response in a mammal which comprises, delivering a polypeptide of the present invention via a vector directing expression of the polynucleotide and coding for

the polypeptide *in vivo* in order to induce such an immunological response to produce antibody to protect said animal from diseases.

It will be appreciated that the present invention therefore provides a method of treating abnormal conditions such as, for instance, cancer and autoimmune diseases, in particular, colorectal cancer, related to either a presence of, an excess of, or an under-expression of, CASB7439 polypeptide activity. Other abnormal conditions related to CASB7439 expression that the invention seeks to treat are chronic lymphocytic leukaemiae and germ cell tumours.

The present invention further provides for a method of screening compounds to identify those which stimulate or which inhibit the function of the CASB7439 polypeptide. In general, agonists or antagonists may be employed for therapeutic and prophylactic purposes for such diseases as hereinbefore mentioned. Compounds may be identified from a variety of sources, for example, cells, cell-free preparations, chemical libraries, and natural product mixtures. Such agonists, antagonists or inhibitors so-identified may be natural or modified substrates, ligands, receptors, enzymes, etc., as the case may be, of the polypeptide; or may be structural or functional mimetics thereof (see Coligan *et al.*, Current Protocols in Immunology 1(2):Chapter 5 (1991)). Screening methods will be known to those skilled in the art. Further screening methods may be found in for example D. Bennett *et al.*, *J Mol Recognition*, 8:52-58 (1995); and K. Johanson *et al.*, *J Biol Chem*, 270(16):9459-9471 (1995) and references therein.

Thus the invention provides a method for screening to identify compounds which stimulate or which inhibit the function of the polypeptide of the invention which comprises a method selected from the group consisting of:

- (a) measuring the binding of a candidate compound to the polypeptide (or to the cells or membranes bearing the polypeptide) or a fusion protein thereof by means of a label directly or indirectly associated with the candidate compound;
- (b) measuring the binding of a candidate compound to the polypeptide (or to the cells or membranes bearing the polypeptide) or a fusion protein thereof in the presence of a labeled competitor;
- (c) testing whether the candidate compound results in a signal generated by activation or inhibition of the polypeptide, using detection systems appropriate to the cells or cell membranes bearing the polypeptide;

(d) mixing a candidate compound with a solution containing a polypeptide of claim 1, to form a mixture, measuring activity of the polypeptide in the mixture, and comparing the activity of the mixture to a standard; or

5 (e) detecting the effect of a candidate compound on the production of mRNA encoding said polypeptide and said polypeptide in cells, using for instance, an ELISA assay.

The polypeptide of the invention may be used to identify membrane bound or soluble receptors, if any, through standard receptor binding techniques known in the art. Well known screening methods may also be used to identify agonists and antagonists of  
10 the polypeptide of the invention which compete with the binding of the polypeptide of the invention to its receptors, if any.

Thus, in another aspect, the present invention relates to a screening kit for identifying agonists, antagonists, ligands, receptors, substrates, enzymes, etc. for polypeptides of the present invention; or compounds which decrease or enhance the  
15 production of such polypeptides, which comprises:

- (a) a polypeptide of the present invention;
  - (b) a recombinant cell expressing a polypeptide of the present invention;
  - (c) a cell membrane expressing a polypeptide of the present invention; or
  - (d) antibody to a polypeptide of the present invention;
- 20 which polypeptide may be that of SEQ ID NO:2 or 3.

It will be readily appreciated by the skilled artisan that a polypeptide of the present invention may also be used in a method for the structure-based design of an agonist, antagonist or inhibitor of the polypeptide, by:

- (a) determining in the first instance the three-dimensional structure of the polypeptide;
- 25 (b) deducing the three-dimensional structure for the likely reactive or binding site(s) of an agonist, antagonist or inhibitor;
- (c) synthesizing candidate compounds that are predicted to bind to or react with the deduced binding or reactive site; and
- (d) testing whether the candidate compounds are indeed agonists, antagonists or  
30 inhibitors.

Gene therapy may also be employed to effect the endogenous production of CASB7439 polypeptide by the relevant cells in the subject. For an overview of gene therapy, see Chapter 20, Gene Therapy and other Molecular Genetic-based Therapeutic

Approaches, (and references cited therein) in Human Molecular Genetics, T Strachan and A P Read, BIOS Scientific Publishers Ltd (1996).

Vaccine preparation is generally described in Pharmaceutical Biotechnology, Vol.61 Vaccine Design - the subunit and adjuvant approach, edited by Powell and Newman, Plenum Press, 1995. New Trends and Developments in Vaccines, edited by Voller *et al.*, University Park Press, Baltimore, Maryland, U.S.A. 1978. Encapsulation within liposomes is described, for example, by Fullerton, U.S. Patent 4,235,877. Conjugation of proteins to macromolecules is disclosed, for example, by Likhite, U.S. Patent 4,372,945 and by Armor *et al.*, U.S. Patent 4,474,757.

The amount of protein in each vaccine dose is selected as an amount which induces an immunoprotective response without significant, adverse side effects in typical vaccinees. Such amount will vary depending upon which specific immunogen is employed. Generally, it is expected that each dose will comprise 1-1000µg of protein, or 2-100µg, or 4-40µg. An optimal amount for a particular vaccine can be ascertained by standard studies involving observation of antibody titres and other responses in subjects. Following an initial vaccination, subjects may receive a boost in about 4 weeks.

“Isolated” means altered “by the hand of man” from the natural state. If an “isolated” composition or substance occurs in nature, it has been changed or removed from its original environment, or both. For example, a polynucleotide or a polypeptide naturally present in a living animal is not “isolated,” but the same polynucleotide or polypeptide separated from the coexisting materials of its natural state is “isolated”, as the term is employed herein.

“Polynucleotide” generally refers to any polyribonucleotide or polydeoxribonucleotide, which may be unmodified RNA or DNA or modified RNA or DNA including single and double stranded regions.

“Variant” refers to a polynucleotide or polypeptide that differs from a reference polynucleotide or polypeptide, but retains essential properties. A typical variant of a polynucleotide differs in nucleotide sequence from another, reference polynucleotide. Changes in the nucleotide sequence of the variant may or may not alter the amino acid sequence of a polypeptide encoded by the reference polynucleotide. Nucleotide changes may result in amino acid substitutions, additions, deletions, fusions and truncations in the polypeptide encoded by the reference sequence, as discussed below. A typical variant of a polypeptide differs in amino acid sequence from another, reference polypeptide.

Generally, differences are limited so that the sequences of the reference polypeptide and the variant are closely similar overall and, in many regions, identical. A variant and reference polypeptide may differ in amino acid sequence by one or more substitutions, additions, deletions in any combination. A substituted or inserted amino acid residue may or may not be one encoded by the genetic code. A variant of a polynucleotide or polypeptide may be a naturally occurring such as an allelic variant, or it may be a variant that is not known to occur naturally. Non-naturally occurring variants of polynucleotides and polypeptides may be made by mutagenesis techniques or by direct synthesis.

"Identity," as known in the art, is a relationship between two or more polypeptide sequences or two or more polynucleotide sequences, as determined by comparing the sequences. In the art, "identity" also means the degree of sequence relatedness between polypeptide or polynucleotide sequences, as the case may be, as determined by the match between strings of such sequences. "Identity" and "similarity" can be readily calculated by known methods, including but not limited to those described in (Computational Molecular Biology, Lesk, A.M., ed., Oxford University Press, New York, 1988; Biocomputing: Informatics and Genome Projects, Smith, D.W., ed., Academic Press, New York, 1993; Computer Analysis of Sequence Data, Part I, Griffin, A.M., and Griffin, H.G., eds., Humana Press, New Jersey, 1994; Sequence Analysis in Molecular Biology, von Heinje, G., Academic Press, 1987; and Sequence Analysis Primer, Gribskov, M. and Devereux, J., eds., M Stockton Press, New York, 1991; and Carillo, H., and Lipman, D., SIAM J. Applied Math., 48: 1073 (1988). Methods to determine identity may be designed to give the largest match between the sequences tested. Methods to determine identity and similarity are codified in publicly available computer programs. Computer program methods to determine identity and similarity between two sequences include, but are not limited to, the GCG program package (Devereux, J., *et al.*, Nucleic Acids Research 12(1): 387 (1984)), BLASTP, BLASTN, and FASTA (Atschul, S.F. *et al.*, J. Molec. Biol. 215: 403-410 (1990). The BLAST X program is publicly available from NCBI and other sources (BLAST Manual, Altschul, S., *et al.*, NCBI NLM NIH Bethesda, MD 20894; Altschul, S., *et al.*, J. Mol. Biol. 215: 403-410 (1990). The well known Smith Waterman algorithm may also be used to determine identity.

The algorithm used may be FASTA. The parameters for polypeptide or polynucleotide sequence comparison using this algorithm include the following:

Gap Penalty:12

Gap extension penalty: 4

Word size: 2, max 6

Parameters for polypeptide sequence comparison with other methods include the following:

1) Algorithm: Needleman and Wunsch, J. Mol Biol. 48: 443-453 (1970)

Comparison matrix: BLOSSUM62 from Hentikoff and Hentikoff, Proc. Natl. Acad. Sci. USA. 89:10915-10919 (1992)

Gap Penalty: 12

Gap Length Penalty: 4

A program useful with these parameters is publicly available as the "gap" program from Genetics Computer Group, Madison WI. The aforementioned parameters are the default parameters for polypeptide comparisons (along with no penalty for end gaps).

Parameters for polynucleotide comparison include the following:

1) Algorithm: Needleman and Wunsch, J. Mol Biol. 48: 443-453 (1970)

Comparison matrix: matches = +10, mismatch = 0

Gap Penalty: 50

Gap Length Penalty: 3

A program useful with these parameters is publicly available as the "gap" program from Genetics Computer Group, Madison WI. The aforementioned parameters are the default parameters for polynucleotide comparisons.

By way of example, a polynucleotide sequence of the present invention may be identical to the reference sequence of SEQ ID NO:1, that is be 100% identical, or it may include up to a certain integer number of nucleotide alterations as compared to the reference sequence. Such alterations are selected from the group consisting of at least one nucleotide deletion, substitution, including transition and transversion, or insertion, and wherein said alterations may occur at the 5' or 3' terminal positions of the reference nucleotide sequence or anywhere between those terminal positions, interspersed either individually among the nucleotides in the reference sequence or in one or more contiguous groups within the reference sequence. The number of nucleotide alterations is



determined by multiplying the total number of nucleotides in SEQ ID NO:1 by the numerical percent of the respective percent identity (divided by 100) and subtracting that product from said total number of nucleotides in SEQ ID NO:1, or:

$$n_n \leq x_n - (x_n \cdot y),$$

5 wherein  $n_n$  is the number of nucleotide alterations,  $x_n$  is the total number of nucleotides in SEQ ID NO:1, and  $y$  is, for instance, 0.70 for 70%, 0.80 for 80%, 0.85 for 85%, 0.90 for 90%, 0.95 for 95%, etc., and wherein any non-integer product of  $x_n$  and  $y$  is rounded down to the nearest integer prior to subtracting it from  $x_n$ . Alterations of a polynucleotide sequence encoding the polypeptide of SEQ ID NO:2 may create nonsense, missense or frameshift mutations in this coding sequence and thereby alter the polypeptide encoded by the polynucleotide following such alterations.

Similarly, a polypeptide sequence of the present invention may be identical to the reference sequence of SEQ ID NO:2, that is be 100% identical, or it may include up to a certain integer number of amino acid alterations as compared to the reference sequence such that the % identity is less than 100%. Such alterations are selected from the group consisting of at least one amino acid deletion, substitution, including conservative and non-conservative substitution, or insertion, and wherein said alterations may occur at the amino- or carboxy-terminal positions of the reference polypeptide sequence or anywhere between those terminal positions, interspersed either individually among the amino acids in the reference sequence or in one or more contiguous groups within the reference sequence. The number of amino acid alterations for a given % identity is determined by multiplying the total number of amino acids in SEQ ID NO:2 by the numerical percent of the respective percent identity (divided by 100) and then subtracting that product from said total number of amino acids in SEQ ID NO:2, or:

$$25 \quad n_a \leq x_a - (x_a \cdot y),$$

wherein  $n_a$  is the number of amino acid alterations,  $x_a$  is the total number of amino acids in SEQ ID NO:2, and  $y$  is, for instance 0.70 for 70%, 0.80 for 80%, 0.85 for 85% etc., and wherein any non-integer product of  $x_a$  and  $y$  is rounded down to the nearest integer prior to subtracting it from  $x_a$ .

30 "Homolog" is a generic term used in the art to indicate a polynucleotide or polypeptide sequence possessing a high degree of sequence relatedness to a subject

sequence. Such relatedness may be quantified by determining the degree of identity and/or similarity between the sequences being compared as hereinbefore described. Falling within this generic term are the terms "ortholog", meaning a polynucleotide or polypeptide that is the functional equivalent of a polynucleotide or polypeptide in another species and "paralog" meaning a functionally similar sequence when considered within the same species.

"CASB7439 polypeptide" as used herein includes but is not limited to isolated polypeptides comprising an amino acid sequence which has at least 70% identity, or at least 80% identity, or at least 90% identity, or at least 95% identity, or at least 97-99% identity, to that of SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:7, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12 or SEQ ID NO:14 each over the entire length of the respective sequence. Such polypeptides include, but are not limited to, those comprising the amino acid of SEQ ID NO:3, SEQ ID NO:7, SEQ ID NO:10 and SEQ ID NO:11.

"Immunogenicity" as used herein means an ability of a compound to induce, enhance or increase antibody production, or induce, enhance or increase a cellular immune response in an animal or immune cell, wherein said compound may be, but is not limited to, a polynucleotide, polypeptide, polypeptides fragment, and variants thereof.

"Immunoresponse" as used herein includes, but is not limited to, a human or animal's ability to produce antibodies or exhibit a immune cell response to an antigen.

The following examples illustrate various aspects of this invention. These examples do not limit the scope of this invention which is defined by the appended claims.

## EXAMPLES

### Example 1

#### **Real-time RT-PCR analysis in matched tumour and normal colon tissues from multiple patients**

Real-time RT-PCR (U. Gibson. 1996. Genome Research: 6,996) is used to compare mRNA transcript abundance of the candidate antigen in matched tumour and normal colon tissues from multiple patients. In addition, mRNA levels of the candidate gene in a panel of normal tissues are also evaluated by this approach.

Total RNA from normal and tumour colon is extracted from snap frozen biopsies using TriPure reagent (Boehringer). Total RNA from normal tissues is purchased from InVitrogen or is extracted from snap frozen biopsies using TriPure reagent. Poly-A+

mRNA is purified from total RNA after DNAase treatment using oligo-dT magnetic beads (Dyna). Quantification of the mRNA is performed by spectrofluorimetry (VersaFluor, BioRad) using SybrII dye (Molecular Probes). Primers for real-time PCR amplification are designed with the Perkin-Elmer Primer Express software using default options for TaqMan amplification conditions.

Real-time reactions are assembled according to standard PCR protocols using 2 ng of purified mRNA for each reaction. SybrI dye (Molecular Probes) is added at a final dilution of 1/75,000 for real-time detection. Amplification (40 cycles) and real-time detection is performed in a Perkin-Elmer Biosystems PE7700 system using conventional instrument settings. Ct values are calculated using the PE7700 Sequence Detector software. Several Ct values are obtained for each samples: for the patient samples, the tumour Ct (CtT) and the matched normal colon Ct (CtN) values on the candidate TAA, and for the panel of normal tissue samples, a CtXY for each normal tissue XY. An another Ct (CtA) is also calculated on Actin gene, as an internal reference, for all of the samples. Alternatively, real-time PCR amplification can be monitored using a Taqman probe. Amplification (40 cycles) and real-time detection is performed in a Perkin-Elmer Biosystems PE7700 system using conventional instrument settings. Ct values are calculated using the PE7700 Sequence Detector Software. Ct values are obtained from each tissue sample for the target mRNA (CtX) and for the actin mRNA (CtA).

As the efficiency of PCR amplification under the prevailing experimental conditions is close to the theoretical amplification efficiency,  $2^{(CtN/T/XY-CtA)}$  value is an estimate of the relative TAA transcript level of the sample, standardised with respect to Actin transcript level. A value of 1 thus suggests the candidate antigen and Actin have the same expression level.

Real-time PCR reactions were first performed on tumour colon and matching normal colon from biopsies of 12 patients (see Table 1). Reactions were then performed on a more complete data set totalling 18 patients (see Table 2) (are included in this data set the first 12 patients). Duplicates for 6 out of these 18 patients were made in this data set. Six further patients were tested, and the results were pooled with the previous 18. The statistics on the final pool are shown in Table 3, and illustrated in Figure 1.

A series of 48 normal tissue samples, representing 29 different tissues, were also tested by the same procedure (analysed normal tissues are given in able 3). TAA transcript levels are calculated as described above. The proportion of patients over-

expressing the candidate antigen, as well as the average transcript over-expression versus normal tissues is also calculated from this data set. The results are illustrated in Figure 1.

5 **Table 1: CASB7439 Real-time PCR expression results: data set of 12 patients.**

% of patients with a mRNA level higher in matched tumour colon (positive patients)	92%
% of patients with a mRNA level at least 3 fold higher in matched tumour colon	92%
% of patients with a mRNA level at least 10 fold higher in matched tumour colon	92%
% of patients with a mRNA level at least 3 fold lower in matched tumour colon.	8%
Average matched normal colon mRNA level (Actin standardised)	0.0026
Average matched tumour colon mRNA level in positive patients (Actin standardised)	0.265
Average mRNA over-expression fold	2028
Median mRNA over-expression fold	115
Average normal tissues mRNA level	0.0079
Median normal tissues mRNA level	0.0016
Average normal tissues mRNA level	0.0064
Median normal tissues mRNA level	0.0017
% of patients with a mRNA level higher than average normal tissues	92%
% of patients with a mRNA level higher than 10 fold average normal tissues	75%
Normal non-dispensable tissues higher than median normal tissue mRNA level	None

**Table 2: CASB7439 Real-time PCR expression results: data set of 18 patients.**

% of patients with a mRNA level higher in matched tumour colon (positive patients)	89%
% of patients with a mRNA level at least 3 fold higher in matched tumour colon	89%
% of patients with a mRNA level at least 10 fold higher in matched tumour colon	78%
% of patients with a mRNA level at least 3 fold lower in matched tumour colon.	5%
Average matched normal colon mRNA level (Actin standardised)	0.005
Average matched tumour colon mRNA level in positive patients (Actin standardised)	0.152
Average mRNA over-expression fold	1100
Median mRNA over-expression fold	60
Average normal tissues mRNA level	0.0065
Median normal tissues mRNA level	0.0015
Average normal tissues mRNA level	0.005
Median normal tissues mRNA level	0.0015
% of patients with a mRNA level higher than median normal tissues	94%
% of patients with a mRNA level higher than 10 fold median normal tissues	94%
Normal non-dispensable tissues higher than median normal tissue mRNA level	None

10

**Table 3: CASB7439 Real-time PCR expression results: data set of 24 patients**

% of patients with a CASB7439 transcript level higher in tumour colon than adjacent normal colon (positive patients)	<b>92%</b>
% of positive patients with a CASB7439 transcript level at least 10 fold higher in tumour colon than adjacent normal colon	<b>75%</b>
Average transcript over-expression fold in tumors of positive patients	<b>1289</b>
% of patients with a CASB7439 transcript level higher in tumour colon than normal tissue average	<b>96%</b>
% of patients with a mRNA level at least 10 fold higher in tumour colon than normal tissue average	<b>62.5%</b>
Normal tissues where CASB7439 transcript expression is equivalent to tumour transcript level in tumours	<b>none</b>

Real-time PCR reactions were also performed using the Taqman protocol ( as described above) on tumour colon and adjacent normal colon from biopsies of 6 patients (see Table 4). Three replicate measures were taken for each, and the average was used for further calculations. Results are shown in figure 1. Moreover, 36 normal tissue samples, representing 28 different tissues (see Table 5), were also tested by the same procedure. Results are shown in Figure 2.

10 **Table 4: CASB7439 Real-time PCR expression results using Taqman probe**

Number of tumor samples from different patients	<b>6</b>
% of patients with a CASB7439 transcript level higher in tumour colon than adjacent normal colon (positive patients)	<b>100%</b>
% of positive patients with a CASB7439 transcript level at least 10 fold higher in tumour colon than adjacent normal colon	<b>83%</b>
Average transcript over-expression fold in tumors of positive patients	<b>109</b>
% of patients with a CASB7439 transcript level higher in tumour colon than normal tissue average	<b>100%</b>
% of patients with a mRNA level at least 10 fold higher in tumour colon than normal tissue average	<b>100%</b>
Normal tissues where CASB7439 transcript expression is equivalent to tumour transcript level in tumours	<b>none</b>

The results clearly suggest CASB7439 transcript is over-expressed in colorectal tumours compared to adjacent normal colon and to all of the above mentioned normal tissues. More than 90% of the patients strongly over-express CASB7439 transcript in tumour, as compared to adjacent normal colon. Average over-expression fold in the tumors is at least of 100. Moreover, more than 90% of the patients over-express the CASB7439 transcript

in colorectal tumors as compared to other normal tissues, more than 60 % of them over-expressing it at least 10 fold.

**Table 5: listing of normal tissues used for CASB7439 transcript expression analysis.**

<b>Tissue</b>	<b>Abbreviation</b>
Adrenal gland	Ad_Gl
Aorta	Ao
Bladder	Bl
Bone marrow	Bo_Ma
Brain	Bra
Cervix	Ce
Colon	Co
Fallopian tube	Fa_Tu
Heart	He
Ileum	Il
Kidney	Ki
Liver	Li
Lung	Lu
Lymph node	Ly_No
Oesophagus	Oe
Parathyroid gland	Pa_Thy
Rectum	Re
Skin	Sk
Skeletal muscle	Sk_Mu
Small intestine	Sm_In
Spleen	Sp
Stomach	St
Thyroid gland	Thy
Trachea	Tra
Ovary	Ov
Placenta	Pl
Prostate	Pr
Testis	Te

5

### Example 2

#### **Differential Screening of cDNA arrays.**

Identification of tumour-associated genes in the subtracted cDNA library is  
 10 accomplished by differential screening.

Total bacterial DNA is extracted from 100 µl over-night cultures. Bacteria are lysed  
 with guanidium isothiocyanate and the bacterial DNA is affinity purified using magnetic  
 glass (Boehringer). Plasmid inserts are recovered from the bacterial DNA by Advantage  
 PCR amplification (Clontech). The PCR products are dotted onto two nylon membranes to  
 15 produce high density cDNA arrays using the Biomek 96 HDRT tool (Beekman). The

spotted cDNA is covalently linked to the membrane by UV irradiation. The first membrane is hybridised with a mixed cDNA probe prepared from the tumour of a single patient. The second membrane is hybridised with an equivalent amount of mixed cDNA probe prepared from normal colon of the same patient. The probe cDNA is prepared by PCR amplification as described above and is labelled using the AlkPhos Direct System (Amersham). Hybridisation conditions and stringency washes are as described in the AlkPhos Direct kit. Hybridized probe is detected by chemiluminescence. Hybridisation intensities for each cDNA fragment on both blots are measured by film densitometry or direct measurement (BioRad Fluor-S Max). The ratio of the tumour to normal hybridisation intensities (T/N) is calculated for each gene to evaluate the degree of over-expression in the tumour. Genes which are significantly over-expressed in colon tumours are followed-up. Significance is arbitrarily defined as one standard deviation of the T/N frequency distribution. Differential screening experiments are repeated using RNA from multiple patient donors (>18) to estimate the frequency of over-expressing tumours in the patient population.

In addition, the DNA arrays are hybridised with mixed cDNA probes from normal tissues other than colon (see list above) to determine the level of expression of the candidate gene in these tissues.

### Example 3

#### **DNA microarrays**

DNA micro-arrays are used to examine mRNA expression profiles of large collections of genes in multiple samples. This information is used to complement the data obtained by real-time PCR and provides an independent measure of gene expression levels in tumors and normal tissues.

Examples of current technologies for production of DNA micro-arrays include 1) The Affymetrix "GeneChip" arrays in which oligonucleotides are synthesized on the surface of the chip by solid phase chemical synthesis using a photolithographic process 2) DNA spotting technology in which small volumes of a DNA solution are robotically deposited and then immobilized onto the surface of a solid phase (e.g. glass). In both instances, the chips are hybridized with cDNA or cRNA which has been extracted from the tissue of interest (e.g. normal tissue, tumour etc...) and labeled with radioactivity or with a fluorescent reporter molecule. The labeled material is hybridized to the chip and the amount of probe bound to each sequence on the chip is determined using a specialized

scanner. The experiment can be set-up with a single fluorescent reporter (or radioactivity) or, alternatively, can be performed using two fluorescent reporters. In this latter case, each of the two samples is labeled with one of the reporter molecules. The two labeled samples are then hybridized competitively to the sequences on the DNA chip. The ratio of the two fluorescent signals is determined for each sequence on the chip. This ratio is used to calculate the relative abundance of the transcript in the two samples. Detailed protocols are available from a number of sources including "DNA Microarrays: A practical approach. Schena M. Oxford University Press 1999" and the World Wide Web (<http://cmgm.stanford.edu/pbrown/protocols/index.html>), <http://arrayit.com/DNA-Microarray-Protocols/>) and specialized distributors (e.g. Affymetrix).

#### Example 5

##### **Northern-Southern blot analysis**

Limited amounts of mixed tumour and matched normal colon cDNA are amplified by Advantage PCR (see above). Messenger RNA from multiple normal tissues is also amplified using the same procedure. The amplified cDNA (1 µg) is electrophoresed on a 1.2% agarose gel and transferred onto a nylon membrane. The membrane is hybridised (AlkPhos Direct System) with a probe prepared using a fragment of the candidate TAA cDNA. Northern-Southern analysis provides information on transcript size, presence of splice variants and transcript abundance in tumour and normal tissues.

#### Example 6

##### **Northern blot analysis**

Northern blots are produced according to standard protocols using 1 µg of poly A+ mRNA. Radioactive probes are prepared using the Ready-to-Go system (Pharmacia).

#### Example 7

##### **Experimental identification of the full length cDNA sequence**

Colon tumour cDNA libraries are constructed using the Lambda Zap II system (Stratagene) from 5 µg of polyA+ mRNA. The supplied protocol is followed except that SuperscriptII (Life Technologies) is used for the reverse transcription step. Oligo dT-primed and random-primed libraries are constructed. About  $1.5 \times 10^6$  independent phages



are plated for each screening of the library. Phage plaques are transferred onto nylon filters and hybridised using a cDNA probe labelled with AlkPhos Direct. Positive phages are detected by chemiluminescence. Positive phage are excised from the agar plat, eluted in 500µl SM buffer and confirmed by gene-specific PCR. Eluted phages are converted to single strand M13 bacteriophage by *in vivo* excision. The bacteriophage is then converted to double strand plasmid DNA by infection of *E. coli*. Infected bacteria are plated and submitted to a second round of screening with the cDNA probe. Plasmid DNA is purified from positive bacterial clones and sequenced on both strands.

When the full length gene cannot be obtained directly from the cDNA library, missing sequence is isolated using RACE technology (Marathon Kit, ClonTech.). This approach relies on reverse transcribing mRNA into double strand cDNA, ligating linkers onto the ends of the cDNA and amplifying the desired extremity of the cDNA using a gene-specific primer and one of the linker oligonucleotides. Marathon PCR products are cloned into a plasmid (pCRII-TOPO, InVitrogen) and sequenced.

The polynucleotide of SEQ ID NO:1 was obtained using this procedure.

#### Example 8.

#### **EST profiles**

A complementary approach to experimental antigen tissue expression characterization is to explore the human EST database. ESTs ('Expressed Sequence Tags') are small fragments of cDNA made from a collection of mRNA extracted from a particular tissue or cell line. Such database currently provides a massive amount of human ESTs ( $2 \times 10^6$ ) from several thousands of cDNA tissue libraries, including tumoral tissues from various types and states of disease. By means of informatics tools (Blast), a comparison search of the CASB7439 sequence is performed in order to have further insight into tissue expression.

**EST distribution of CASB7439:**

<b>EST GenBank Accession number</b>	<b>EST cDNA tissue library</b>
C00634	Human adult (K.Okubo)
AA468668	NCI_CGAP_Co3
AA565752	NCI_CGAP_Co11
AA565766	NCI_CGAP_Co11
AA565767	NCI_CGAP_Co11
AI337239	NCI_CGAP_Co16
AI337448	NCI_CGAP_Co16
AI393930	NCI_CGAP_CLL1
AI473673	NCI_CGAP_Co14
AI632444	NCI_CGAP_GC6
AI861937	NCI_CGAP_Co16
AI825214	NCI_CGAP_GC6
AW080652	NCI_CGAP_Co19
AW083899	NCI_CGAP_Co19
AW206058	NCI_CGAP_Sub3
AW237006	NCI_CGAP_GC6
AW364626	DT0036
AW449612	NCI_CGAP_Sub5

These ESTs match perfectly with CASB7439. The list contains 9 ESTs from 4  
5 different tumor colon libraries, one EST from one normal colon library, 3 ESTs from one  
tumor germ cell library, one EST from one chronic lymphocyte leukemia cells library, 2  
ESTs from 2 mixed tumors libraries, 2 ESTs from libraries of unknown type. These  
results clearly suggests, as expected, that CASB7439 is over-expressed in tumor tissues,  
as compared to normal tissues.

**Example 9:****9.1 Expression and purification of tumour-specific antigens**

Expression in microbial hosts, or alternatively *in vitro* transcription/translation, is  
used to produce the antigen of the invention for vaccine purposes and to produce protein  
15 fragments or whole protein for rapid purification and generation of antibodies needed for  
characterization of the naturally expressed protein by immunohistochemistry or for  
follow-up of purification.

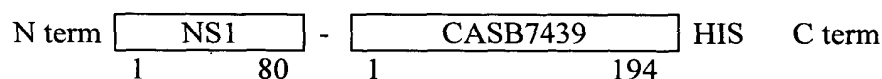
Recombinant proteins may be expressed in two microbial hosts, *E. coli* and in  
yeast (such as *Saccharomyces cerevisiae* or *Pichia pastoris*). This allows the selection of  
20 the expression system with the best features for this particular antigen production. In

As mentioned above, several constructs might undergo comparative evaluation:

Therefore, two constructs are proposed:

**Construct 2:** Full length mutated CASB7439 cDNA in fusion with NS1 cDNA as EFP and with a histidine tail coding cDNA as an AFP (SEQ ID NO:9). It is proposed in this construct to have the first 50 codons of native CASB7439 cDNA replaced by codons specific of the *E. coli* codon usage, to enhance expression potential of CASB7439 in its *E. coli* host. The encoded fusion protein sequence is SEQ ID NO:10.

The CASB7439 protein design is as shown below:



“NS1” is the N-terminal fragment (80 amino acids) of the Influenza protein NS1.  
“HIS” is a polyhistidine tail.

The recombinant strain used is AR58: a cryptic  $\lambda$  lysogen derived from N99 that is gal E::Tn 10, $\Delta$ -8(chlD-pgl), $\Delta$ -H1(cro-chlA),N+, and cI857 (*Proc.Natl.Acad.Sci.USA* vol82, pp.88-92, January 1985 Biochemistry)

When the recombinant strains are available, the recombinant product is characterized by the evaluation of the level of expression and the prediction of further solubility of the protein by analysis of the behavior in the crude extract.

After growth on appropriate culture medium and induction of the recombinant protein expression, total extracts are analyzed by SDS-PAGE. The recombinant proteins are visualized in stained gels and identified by Western blot analysis using specific antibodies.

Plasmid:

10	name: TCM 281	pRIT..15143
	replicon: pMB1	
	selection: Kan	
	promotor: PL long	
	insert: NS1-C74-39-His	

15 Expression of the recombinant protein from construct 1:

Bacteria was grown in LB medium + 50 µg/ml Kan at 30°C

When the culture reached OD = 0.5 (620nm), the culture was heated up to 39°C, after 5 hours of induction, cells were harvested

Extract preparation:

20	Cell concentration:	.50X.. in buffer PBS + complete...
	Disruption:	press french 3X
	Centrifugation:	30 minutes at 14000t
	Comment:	>90% in the supernatant of cellular extract

25 The cell extract was run on a 12.5% SDS PAGE, and subsequently stained with Coomassie blue. A Western blot was also performed using an commercial monoclonal antibody against the poly-histidine tail (Quiagen). The resulting gels (Figures 3 and 4), show that the protein is expressed, and visible in the cell extract supernatant.

The purification scheme follows a classical approach based on the presence of an His affinity tail in the recombinant protein. In a typical experiment the disrupted cells are filtered and the acellular extracts loaded onto an Ion Metal Affinity Chromatography (IMAC; Ni<sup>++</sup>NTA from Qiagen) that will specifically retain the recombinant protein.

5 The retained proteins are eluted by 0-500 mM imidazole gradient (possibly in presence of a detergent) in a phosphate buffer.

The supernatant from the harvested culture was denatured in 6M urea, 100mM NaH<sub>2</sub>PO<sub>4</sub>, 10mM Tris, PH 8, and loaded on a chromatographic column IMAC Qiagen NTA Ni<sup>++</sup> under the following conditions:

10

Equilibration buffer:	NaH <sub>2</sub> PO <sub>4</sub>	100mM	PH 8
	Tris	10mM	
	Urea	6M	

15

Sample: supernatant in urea 6M, 100mM NaH<sub>2</sub>PO<sub>4</sub>, 10mM Tris

Wash buffers:	1) NaH <sub>2</sub> PO <sub>4</sub>	100mM	PH 8
	Tris	10mM	
	urea	6 M	
	Imidazole	25mM	

20

	2) NaH <sub>2</sub> PO <sub>4</sub>	100mM	PH 8
	Tris	10mM	
	Urea	6mM	
	Imidazole	50mM	

25

Elution buffer:	NaH <sub>2</sub> PO <sub>4</sub>	100mM	PH 5,5
	Tris	10mM	
	Urea	6M	
	Imidazole	500mM	

30

The eluted protein in 500mM imidazole + 6M urea is dialysed under the following conditions:

- PBS PH 7,2 + sarkosyl 0.5% + 4M urea
- idem at 2M urea 2 hours
- idem at 0M urea 2 hours

5

The final material is frozen and stored. The protein content was quantified using a Lowry protein assay (0.9 mg/1.2 mL). The purity was assessed by a 12.5% PAGE SDS stained with Coomassie blue (Figure 5), and the presence of the recombinant protein was checked by Western blot, using a anti-polyhistidine monoclonal antibody (Figure 6)

10

A comparative evaluation of the different versions of the expressed antigen will allow the selection of the most promising candidate that is to be used for further purification and immunological evaluation.

## 9.2 Antibody production and immunohistochemistry

15

Small amounts of relatively purified protein can be used to generate immunological tools in order to

- a) detect the expression by immunohistochemistry in normal or cancer tissue sections;
- b) detect the expression, and to follow the protein during the purification process (ELISA/ Western Blot); or
- c) characterize/ quantify the purified protein (ELISA).

20

### 9.2.1 Polyclonal antibodies:

#### Immunization

Rabbits are immunized, intramuscularly (I.M.), 3 times at 3 weeks intervals with 100µg of protein, formulated in the adjuvant 3D-MPL/QS21. Three weeks after each immunization a blood sample is taken and the antibody titer estimated in the serum by ELISA using the protein as coating antigen following a standard protocol.

25

#### ELISA

30

96 well microplates (maxisorb Nunc) are coated with 5µg of protein overnight at 4°C. After 1hour saturation at 37°C with PBS NCS 1%, serial dilution of the rabbit sera is added for 1H 30 at 37°C (starting at 1/10). After 3 washings in PBS Tween, anti rabbit biotinylated anti serum (Amersham ) is added (1/5000). Plates are washed and

peroxydase coupled streptavidin (1/5000) is added for 30 minutes at 37°C. After washing, 50µl TMB (BioRad) is added for 7 minutes and the reaction then stopped with H<sub>2</sub>SO<sub>4</sub> 0.2M. The OD can be measured at 450 nm and midpoint dilutions calculated by SoftmaxPro.

5

### 9.2.2 Monoclonal antibodies:

#### Immunization

5 BALB/c mice are immunized 3 times at 3 week intervals with 5 µg of purified protein. Bleedings are performed 14 days post II and 1 week post 3. The sera are tested by Elisa on purified protein used as coated antigen. Based on these results (midpoint dilution >10000 ) one mouse is selected for fusion.

10

#### Fusion/ HAT selection

Spleen cells are fused with the SP2/0 myeloma according to a standard protocol using PEG 40% and DMSO 5%. Cells are then seeded in 96 well plates 2.5 x10<sup>4</sup> - 10<sup>5</sup> cells/well and resistant clones will be selected in HAT medium. The supernatant of these hybridomas will be tested for their content in specific antibodies and when positive, will be submitted to 2 cycles of limited dilution . After 2 rounds of screening, 3 hybridomas will be chosen for ascitis production.

20

### 9.2.3 Immunohistochemistry

When antibodies are available, immuno staining is performed on normal or cancer tissue sections, in order to determine:

- the level of expression of the antigen of the invention in cancer relative to normal tissue or
- the proportion of cancer of a certain type expressing the antigen
- if other cancer types also express the antigen
- the proportion of cells expressing the antigen in a cancer tissue

25

### 30 Tissue sample preparation

After dissection, the tissue sample is mounted on a cork disk in OCT compound and rapidly frozen in isopentane previously super cooled in liquid nitrogen (-160°C). The

block will then be conserved at -70°C until use. 7-10µm sections will be realised in a cryostat chamber (-20, -30°C).

### Staining

5 Tissue sections are dried for 5 minutes at room temperature ("RT"), fixed in acetone for 10 minutes at RT, dried again, and saturated with PBS 0.5% BSA 5% serum. After 30 minutes at RT either a direct or indirect staining is performed using antigen specific antibodies. A direct staining leads to a better specificity but a less intense staining whilst an indirect staining leads to a more intense but less specific staining.

10

### **9.3 Analysis of human cellular immune responses to the antigen of the invention**

The immunological relevance of the antigen of the invention can be assessed by *in vitro* priming of human T cells. All T cell lymphocyte lines and dendritic cells are derived from PBMCs (peripheral blood mononuclear cells) of healthy donors (HLA-A2  
15 subtype). An HLA-A2.1/Kb transgenic mouse model is also used for screening of HLA-A2.1 peptides.

Newly discovered antigen-specific CD8<sup>+</sup> T cell lines are raised and maintained by weekly *in vitro* stimulation. The lytic activity and the γ-IFN production of the CD8<sup>+</sup> lines in response to the antigen or antigen derived-peptides is tested using standard procedures.

20 Two strategies to raise the CD8<sup>+</sup> T cell lines are used: a peptide-based approach and a whole gene-based approach. Both approaches require the full-length cDNA of the newly discovered antigen in the correct reading frame to be either cloned in an appropriate delivery system or to be used to predict the sequence of HLA binding peptides.

25

#### Peptide-based approach

Briefly, transgenic mice are immunized with adjuvanted HLA-A2 peptides, those unable to induce a CD8<sup>+</sup> response (as defined by an efficient lysis of peptide-pulsed autologous spleen cells) will be further analyzed in the human system.

30 Human dendritic cells (cultured according to Romani *et al.*) will be pulsed with peptides and used to stimulate CD8<sup>+</sup> -sorted T cells (by Facs). After several weekly stimulations, the CD8<sup>+</sup> lines will be first tested on peptide-pulsed autologous BLCL (EBV-B transformed cell lines). To verify the proper *in vivo* processing of the peptide,



the CD8<sup>+</sup> lines will be tested on cDNA-transfected tumour cells (HLA-A2 transfected LnCaP, Skov3 or CAMA tumour cells).

#### Whole gene-based approach

5 CD8<sup>+</sup> T cell lines will be primed and stimulated with either gene-gun transfected dendritic cells, retrovirally transduced B7.1-transfected fibroblasts, recombinant pox virus or adenovirus infected dendritic cells. Virus infected cells are very efficient to present antigenic peptides since the antigen is expressed at high level but can only be used once to avoid the over-growth of viral T cells lines.

10 After alternated stimulations, the CD8<sup>+</sup> lines are tested on cDNA-transfected tumour cells as indicated above. Peptide specificity and identity is determined to confirm the immunological validation.

#### CD4<sup>+</sup> T-cell response

15 Similarly, the CD4<sup>+</sup> T-cell immune response can also be assessed. Generation of specific CD4<sup>+</sup> T-cells is made using dendritic cells loaded with recombinant purified protein or peptides to stimulate the T-cells.

#### Predicted epitopes (nonamers and decamers) binding HLA alleles:

20 The HLA Class I binding peptide sequences are predicted either by the Parker's algorithm (Parker, K. C., M. A. Bednarek, and J. E. Coligan. 1994. Scheme for ranking potential HLA-A2 binding peptides based on independent binding of individual peptide side-chains. J. Immunol. 152:163 and [http://bimas.dcrt.nih.gov/molbio/hla\\_bind/](http://bimas.dcrt.nih.gov/molbio/hla_bind/)) or the Rammensee method (Rammensee, Friede, Stevanovic, MHC ligands and peptide motifs: 1st listing, Immunogenetics 41, 178-228, 1995; Rammensee, Bachmann, Stevanovic: 25 MHC ligands and peptide motifs. Landes Bioscience 1997, and <http://134.2.96.221/scripts/hlaserver.dll/home.htm>). Peptides are then screened in the HLA-A2.1/Kb transgenic mice model (Vitiello *et al.*).

30 The HLA Class II binding peptide sequences are predicted using the Tepitope algorithm, with a score cut-off set to 6 (Sturniolo, Hammer *et al.*, Nature Biotechnology. 1999. 17;555-561).

The following tables gather the Class I and II predicted epitope sequences:

HLA-A 0201: decamers				
Rank	Start Position	Subsequence Residue Listing	Parker's Score °	SEQ ID:
1	64	KLVNLTGFQAL	142.060	SEQ ID NO:16

°: Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence.

HLA-A 0201: nonamers				
Rank	Start Position	Subsequence Residue Listing	Parker's Score °	SEQ ID:
1	182	ELLDFSSWL	507.976	SEQ ID NO:17
2	104	RLLAEHDAV	126.098	SEQ ID NO:18
3	64	KLVNLTGFQA	100.850	SEQ ID NO:19

°: Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence.

HLA-A 24: nonamers				
Rank	Start Position	Subsequence Residue Listing	Parker's Score	SEQ ID:
1	97	EYIRALQRL	360.000	SEQ ID NO:20

5 °: Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence.

HLA-A 24: decamers				
Rank	Start Position	Subsequence Residue Listing	Parker's Score	SEQ ID:
1	97	EYIRALQRL	360.000	SEQ ID NO:21

°: Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence.

HLA-B 7: decamers				
Rank	Start Position	Subsequence Residue Listing	Parker's Score	SEQ ID:
1	111	AVRNALAGGL	600.000	SEQ ID NO:22

°: Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence.

10

HLA-B 4403: decamers				
Rank	Start Position	Subsequence Residue Listing	Parker's Score	SEQ ID:
1	156	SEPGSPRSAY	360.000	SEQ ID NO:23
2	89	VETLRSAVEY	180.000	SEQ ID NO:24

°: Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence.

HLA-DRB1*1501: nonamers				
Rank	Start Position	Subsequence Residue Listing	Tepitope Score	SEQ ID:
1	99	IRALQRLLA	5.6	SEQ ID NO:25

HLA-DRB1*1502: nonamers				
Rank	Start Position	Subsequence Residue Listing	Tepitope Score	SEQ ID :
1	99	IRALQRLLA	4.6	SEQ ID NO:25

HLA-DRB1*0402: nonamers				
Rank	Start Position	Subsequence Residue Listing	Tepitope Score	SEQ ID:
1	120	LRPQAVRPS	5.4	SEQ ID NO:26

HLA-DRB1*1101: nonamers				
Rank	Start Position	Subsequence Residue Listing	Tepitope Score	SEQ ID:
1	99	IRALQRLLA	4.8	SEQ ID NO:25

HLA-DRB1*1102: nonamers				
Rank	Start Position	Subsequence Residue Listing	Tepitope Score	SEQ ID:
1	120	LRPQAVRPS	6.2	SEQ ID NO:26

5

HLA-DRB1*1104: nonamers				
Rank	Start Position	Subsequence Residue Listing	Tepitope Score	SEQ ID:
1	99	IRALQRLLA	5.8	SEQ ID NO:25

HLA-DRB1*1106: nonamers				
Rank	Start Position	Subsequence Residue Listing	Tepitope Score	SEQ ID:
1	99	IRALQRLLA	5.8	SEQ ID NO:25

HLA-DRB1*1301: nonamers				
Rank	Start Position	Subsequence Residue Listing	Tepitope Score	SEQ ID:
1	120	LRPQAVRPS	6.6	SEQ ID NO:26
2	73	LRQHVPHGG	4.9	SEQ ID NO:27
3	31	LLRCSRRRR	4.4	SEQ ID NO:33

HLA-DRB1*1302: nonamers				
Rank	Start Position	Subsequence Residue Listing	Tepitope Score	SEQ ID:
1	120	LRPQAVRPS	5.6	SEQ ID NO:26

HLA-DRB1*1304: nonamers				
Rank	Start Position	Subsequence Residue Listing	Tepitope Score	SEQ ID:
1	120	LRPQAVRPS	6.2	SEQ ID NO:26
2	73	LRQHVP HGG	4.8	SEQ ID NO:27
3	31	LGFQALRQH	4.6	SEQ ID NO:28

HLA-DRB1*1305: nonamers				
Rank	Start Position	Subsequence Residue Listing	Tepitope Score	SEQ ID:
1	99	IRALQRLLA	4.8	SEQ ID NO:25

HLA-DRB1*0703: nonamers				
Rank	Start Position	Subsequence Residue Listing	Tepitope Score	SEQ ID:
1	112	VRNALAGGL	5.1	SEQ ID NO:29
2	98	YIRALQRL	4.8	SEQ ID NO:30
3	65	LVNLGFQAL	4.5	SEQ ID NO:31

HLA-DRB5*0101: nonamers				
Rank	Start Position	Subsequence Residue Listing	Tepitope Score	SEQ ID:
1	96	VEYIRALQR	4.3	SEQ ID NO:32

5

Example 10**CASB7439 specific cellular immune response**

A further way of assessing CASB7439 immunogenicity is to demonstrate CASB7439 antigen has a potential to trigger a cellular immune response. For that purpose, it has to be verified the human CD4+T-cell repertoire has the ability to recognise CASB7439 antigens presented by APC's (antigen-presenting cells) in a MHC class II restricted manner.

10

To demonstrate CASB7439 antigen can generate a specific CD4+T-cell activity in human, as well as to identify CASB7439 epitopes, a series of "*in vitro* priming" experiments have been carried out with the PBMC's of three healthy donors.

#### 5 *In vitro* Priming of donor #1

*In-vitro* priming cultures with the PBMC of donor #1 were established using 15-mer peptides overlapping by 11 amino acids from the sequence of CASB7439.

Peptides were combined in pools of 6 or 7 peptides/pool (the resulting 45 peptide sequences and 7 pools are detailed in table 5), and pulsed onto autologous dendritic cells (DCs). Following 4 stimulation cycles, donor #1 PBMC cell lines were assayed for proliferation by using a 3H-thymidine incorporation assay and for IFN- $\gamma$  synthesis by ELISA.

A number of positive peptide pools were identified, with 21 and 7 cells lines exhibited stimulation index (S.I.)>3 and S.I.>5, respectively (Stimulation index reflects the ratio of activity from T cells incubated with DC pulsed with relevant vs. irrelevant peptide or protein).

All positive 21 lines were further re-stimulated with individual peptides. One line, designated 3H8, showed a specific reactivity to peptide #21.

To map the particular epitope within peptide 21 and recognised by the T-cells of donor #1, T-Cell clones ere individualized from the T-cell lines. For this purpose, the line 3H8 was re-stimulated on antigen expanded using polyclonal activator PHA, and cloned on PHA. Clones were then tested for peptide stimulation in IFN- $\gamma$  ELSA assays.

Several clones from line 3H8 were shown to recognize peptide 21. Clones generated from this 3H8 line recognised peptide but failed to recognise *E. coli*-derived NS1-CASB7439 protein. Therefore a similar *in vitro* priming procedure with a new donor has been undertaken to generate T-clones able to recognise the whole CASB7439 protein presented by APC's.

#### *In vitro* Priming of donor #2

*In vitro* priming experiments were performed with the PBMC from an additional donor #2 in similar experimental condition as donor #1. In brief, PBMC were stimulated with autologous DC pulsed with the 7 pools of 6-7 peptides at a concentration of 250 ng/ml for each peptide. 29 cells lines showed reactivity to pooled peptides, and were

further assayed on individual peptides (at 250 ng/ml) and on *E. coli*-derived NS1-CASB7439 protein (10 µg/ml).

5 5 of these lines (lines 3A3, 4C5, 3C9, 4D5, and 4B12) demonstrated specific reactivity to a particular peptide pool and to both an individual peptide derived from that pool and *E. coli*-derived NS1-CASB7439.

Two of the lines, 3A3 and 4C5, have been cloned using PHA. The resulting clones were assayed against DCs pulsed with *E. coli*-derived NS1-CASB7439 fusion protein (2.5 µg /ml) or with irrelevant protein (OspA: 2.5 µg /ml) and assayed for proliferation (3H-Thy) as well as IFN-γ production. 61 clones from the two cell lines  
10 were shown to recognise *E. coli*-derived protein, and all of the 61 clones have been also shown to recognise the peptide 16 from the pool.

These 16 clones were further characterized by demonstrating MHC Class II restriction. CD4+clones derived from line 3A3 were assayed against DCs pulsed with *E. coli*-derived NS1-CASB7439 protein or irrelevant protein (OspA: 1 µg /ml) in the  
15 presence or absence of antibodies (25 µg /ml) to Class I (W632), Class II (HB145), HLA-DR (L243), or HLA-DQw3 (HB144). These T cell clones were shown to be restricted by MHC Class II, and more precisely are not DR DQw3 restricted. Moreover, a preliminary donor mismatch analyses suggest that these clones are likely restricted by HLA-DQ0602.

Specificity of the NS1-CASB7439 fusion protein generated CD4+ T cell activity  
20 is further demonstrated: indeed, a line 3A3 clone CD4 response titers out with CASB7439 protein. No response to OspA, an irrelevant protein used as negative control, is observed.

### *In vitro* Priming of donor #3

25 *In vitro* priming cultures were established from an additional donor using the same pools of 15-mer peptide overlapping 11 amino acids and the same procedures. Four CD4+lines, including 4A7 and 4E4, demonstrated *E. coli*-derived recombinant protein reactivity that was blocked by antibody to MHC Class II.

Furthermore, CD4+ clones derived from lines 4A7 and 4E4 were assayed against  
30 dendritic cells pulsed with *E. coli*-derived NS1-CASB7439 protein or irrelevant protein (OspA: 10 µg /ml), or CASB7439 peptides (250 ng/ml) in the presence or absence of antibodies to Class I (W632:25 µg /ml) or anti-HLA-DR (L243:25 µg /ml). CD4+ clones derived from these lines present a specificity that is different from the clones described

above, as they are HLA-DR restricted. Moreover, 3 other peptides were shown to be recognised by CD4+T cells (peptides 23, 24, and 25), the total number recognised peptide that were identified being 5 so far.

## 5 Example 11

### **Immunohistochemical analysis of CASB7439 on tumour and normal colon biopsies.**

CASB7439 protein over-expression in colon tumour was verified by immunohistochemistry (IHC) using a CASB7439 specific rabbit polyclonal antibody (Ab CASB7439 #599, 1/50 dilution) directed against an affinity purified CASB7439  $\alpha$ -peptide (SB599  $\alpha$ -peptide, amino acids 1 to 14 of CASB7439).

Ab CASB7439 #599 was generated as follows: a rabbit is immunized with SB599 synthetic  $\alpha$ -peptide that is conjugated to a carrier protein (LKH). Conjugate is formulated with Freund's adjuvant, and two rabbits are immunized with formulated conjugate. Four weeks after the second immunization and four weeks after the third immunization, blood samples are taken. Anti-CASB7439 antibody titers are estimated in the serum by ELISA.

For IHC, paraffin-embedded formalin fixed tissue was sliced into 8 micron sections. Steam heat induced epitope retrieval (SHIER) in 0.1 M sodium citrate buffer (pH 6.0) was used for optimal staining conditions. Sections were incubated with 10% serum/PBS for 5 minutes. Ten micrograms/ml of primary antibody (SB599) was added to each section for 25 minutes followed by a 25 minute incubation with a biotinylated anti-rabbit antibody. Endogenous peroxidase activity was blocked by three 1.5 minute incubations with hydrogen peroxidase. The avidin biotin complex/horse radish peroxidase (ABV/HRP) system was used along with DAB chromogen to visualise antigen expression. Slides were counterstained with hematoxylin.

Figure 7 and 8 shows IHC results on colon tumour #9476 biopsy and colon normal mucosa #9476, respectively. Anti-CASB7439 immunoreactivity was observed at high level in colon cancer and in normal colon at very low level. Anti-CASB7439 immunoreactivity was localised to the cytoplasm and associated with the plasma membrane of the cells.

Example 12**Demonstration of CASB7439 *in vivo* immunogenicity.**

Besides being highly tumour specific, the second critical criterion for a candidate vaccine evaluation is its immunogenicity. One way of assessing the immunogenicity of a protein is to immunize naïve animals with synthetic peptides derived from the antigen sequence, and which reproduce natural epitopes. The generated anti-peptide antibodies will then tend to recognise the native antigen, therefore, showing a specific immune response can be raised against the candidate vaccine antigen.

Because of the very nature of the immune response, a classical dose range analysis is replaced by repeated immunizations, allowing comparison of antibody titers before immunization (control), and after cumulative injections.

Two peptides were selected from CASB7439 antigen sequence for their immunogenic potential: peptides spanning from amino-acids 1-14 (peptide 1) and amino-acids 157-172 (peptide 2). Two rabbits were immunized with each peptide, rabbits SB598 and SB599 with peptide 1 and rabbits SB600 and SB601 with peptide 2.

The selected peptides were conjugated to a carrier protein (KLH). Rabbits were intramuscularly immunized with CASB7439 peptide, 3 times at 3 to 4 weeks intervals with 200 µg of conjugate formulated with Freund's adjuvant. Four weeks after the second immunization (PP) and four weeks after the third immunization (GP), blood samples were taken, and the CASB7439 specific antibody titers were estimated in the serum by ELISA. Thus, a dose range of 0, 200 and 400 µg of the antigen conjugate is reproduced.

ELISAs were done in triplicate for each immunizing peptide and rabbit serum, and performed as follows: 96 well microplates were coated at 4 °C during 16 hours with either 100 ng of CASB7439 peptide or 100 ng of KLH as coating antigens. After 2-hour saturation at 25 °C with BSA (1mg/ml), serial dilution of the rabbit sera was added for 2 hours at 25 °C (starting at dilution 1/100). Anti rabbit antibody, conjugated with universal-HRP, was then added at dilution 1/1000 for 2 hours at 25 °C as secondary antibody. Plates were washed and OPD (0.4 mg/ml) is added for 30 minutes at 25 °C. Reaction was stopped with H<sub>2</sub>SO<sub>4</sub>4M, and OD measured at 492 nm.

ELISA results clearly show an antibody immune response against distinct CASB7439 synthetic peptide can be raised in several rabbits in a dose-dependant manner. This suggests CASB7439 candidate antigen is indeed immunogenic and, when properly



formulated with adjuvant as a vaccine, CASB7439 vaccine is able to induce a strong and specific antibody immune response.

### Example 13

#### 5      **Real-time RT- PCR analysis in a panel of tumour and matched normal tissues of breast from multiple patient samples**

Real-time RT-PCR (U. Gibson. 1996. Genome Research: 6,996) is used to compare mRNA transcript abundance of the candidate antigen in a panel of tumour and matched normal tissues of breast from multiple patient samples. In addition, mRNA levels of the candidate antigen in different normal and tumour cell lines of breast, in a  
10      panel of normal tissues, and finally in breast tumours grown *in vivo* in SCID mice are also evaluated by this approach. This analysis establishes the breast tumour specificity of CASB7439 expression, which is an important criterion that a good antigen candidate must fulfil.

cDNA was derived from prostate, ovarian, colon and breast tumour cell lines as  
15      well as breast tumours, normal breast and other normal tissues. Human tumour explants from breast cancer patients ( $\sim 1\text{mm}^3$ ) are implanted subcutaneously in SCID mice. Time-release hormone pellets ( $\beta$ -estradiol) are co-implanted subcutaneously. When tumours reach  $\sim 5\%$  of the total mouse body weight they are excised and a portion ( $\sim 1\text{mm}^3$ ) is passaged into another set of SCID mice. This process can be repeated indefinitely. SCID-  
20      derived tumour specimens are designated by the passage number followed by 'p'. Real time PCR analysis was performed on matched pairs (early passage/late passage) of SCID-derived human tumours

Total RNA is extracted from snap frozen biopsie or cell lines using TriPure reagent (Boehringer). Poly-A+ mRNA is purified from total RNA after DNAase  
25      treatment using oligo-dT magnetic beads (Dyna). Quantification of the mRNA is performed by spectrofluorimetry (VersaFluor, BioRad) using SybrII dye (Molecular Probes).

Primers for real-time PCR amplification are designed with the Perkin-Elmer Primer Express software using default options for TaqMan amplification conditions. Real  
30      time PCR was performed with CASB7439 specific probes and the expression levels in the tissues determined: 2 distinct TaqMan primer pairs are designed (one pair in

CASB7439 3' UTR region and one pair in CASB7439 ORF region) and used in subsequent real-time RT-PCR analysis.

Real-time reactions are assembled according to standard PCR protocols using 2 ng of reverse transcribed mRNA (Expand RT, Roche) for each reaction. SybrI dye (Molecular Probes) is added at a final dilution of 1/75000 for real-time detection. Amplification (40 cycles) and real-time detection is performed in a Perkin-Elmer Biosystems PE7700 system using conventional instrument settings. Ct values are calculated using the PE7700 Sequence Detector software, and results are standardised with respect to Actin.

Figure 9 demonstrates while almost all colon cell lines expressed CASB7439 so did the breast tumour cell lines MCF-7, MDA-MB415, BT474 and T47D. These results on a panel of cell lines suggest that, in addition to colon cancer, CASB7439 transcript is also surprisingly over-expressed in breast tumour cell lines, as compared to other normal tissues. Expression is also seen in pooled breast tumour tissue, SCID-derived breast tumour 9987 (both passages) and 9077 (late passage only), as shown in Figures 10 and 11. Primers derived from the CASB7439 ORF as well as the 3' end yielded similar profiles. Figures 12-14 confirms breast tumour specificity of CASB7439 expression on an extended panel of breast tumours and normal tissues. Taken together, these real-time RT-PCR results clearly indicate CASB7439 is over-expressed in an majority of breast tumours, as compared to normal tissues.

#### Example 14

#### **Expression and purification of tumour-specific antigens**

##### **14.1 Expression of the present invention antigen in microbial hosts.**

Expression in microbial hosts, or alternatively *in vitro* transcription/translation, is used to produce the antigen of the invention for vaccine purposes and to produce protein fragments or whole protein for rapid purification and generation of antibodies needed for characterization of the naturally expressed protein by western blot, immunohistochemistry or for follow-up of purification.

Recombinant proteins may be expressed in two microbial hosts, *E. coli* and in yeast (such as *Saccharomyces cerevisiae* or *Pichia pastoris*). The use of two expression hosts allows the selection of the expression system with the best features for this

particular antigen production. In general, the recombinant antigen will be expressed in *E. coli* and the reagent protein expressed in yeast.

The expression strategy first involves the design of the primary structure of the recombinant antigen. In general, an expression fusion partner (EFP) is placed at the N terminal extremity to improve levels of expression that could also include a region useful for modulating the immunogenic properties of the antigen, an immune fusion partner (IFP). In addition, an affinity fusion partner (AFP) useful for facilitating further purification is included at the C-terminal end. Moreover, polynucleotide sequence of the coding region of the antigen can also be modified to optimise its expression. Using a codon usage that is adapted to the chosen recombinant expression host can dramatically increase antigen expression yields.

A comparative evaluation of the different versions of the expressed antigen will allow the selection of the most promising candidate that is to be used for further purification and immunological evaluation.

Several constructs underwent comparative evaluation. For rapid expression and purification as well as generation of antibodies against CASB7439, it is proposed to generate in *E. coli* a full length CASB7439 protein with *Influenza* protein NS1 as EFP and a histidine tail as AFP.

Five constructs have been designed:

**Construct 1:** Full length wild type CASB7439 cDNA in fusion with the N terminal fragment (the first 80 amino acids) of *Influenza* protein NS1 coding cDNA as EFP and with a histidine tail coding cDNA as an AFP (SEQ ID NO:8). The encoded fusion protein sequence is SEQ ID NO:10. The CASB7439 protein design is shown in Figure 15.

**Construct 2:** Full length mutated CASB7439 cDNA in fusion with the N terminal fragment of *Influenza* protein NS1 coding cDNA as EFP and with a histidine tail coding cDNA as an AFP (SEQ ID NO:9). This construct was designed in order to comprise the first 50 codons of native CASB7439 cDNA replaced by codons specific of the *E. coli* codon usage, to enhance expression potential of CASB7439 in its *E. coli* host. Only CASB7439 codons that are known to be problematic in *E. coli* host by the man skilled in

the art were replaced by an *E. coli* codon. For each CASB7439 problematic codon, the *E. coli* codon is chosen to encode for the same amino acid and to have a low GC content. The encoded fusion protein sequence is SEQ ID NO:10.

- 5    **Construct 3:** Full length wild type CASB7439 cDNA in fusion with a 11 amino acids T7•Tag<sup>®</sup> (Novagen, Madison, WI) coding cDNA as EFP and with a histidine tail coding cDNA as an AFP (SEQ ID NO:34). The encoded fusion protein is SEQ ID NO: 35.

- 10    **Construct 4:** Full length mutated CASB7439 cDNA in fusion with a 11 amino acids T7•Tag<sup>®</sup> (Novagen, Madison, WI) coding cDNA as EFP and with a histidine tail coding cDNA as an AFP (SEQ ID NO: 36). The first 50 codons of native CASB7439 cDNA replaced by codons specific of the *E. coli* codon usage. The same substitutions as those performed in construct 2 have been carried out in construct 4. The encoded fusion protein sequence is SEQ ID NO:35.

- 15    **Construct 5:** Full length mutated CASB7439 cDNA is fusion with a 11 amino acids T7•Tag<sup>®</sup> (Novagen, Madison, WI) coding cDNA as EFP and a histidine tail coding cDNA as AFP (SEQ ID NO:37). This construct also has the first 50 codons of native CASB7439 cDNA replaced by codons specific of the *E. coli* codon usage. All  
20    CASB7439 codons are modified in this construct to have specific *E. coli* codons and to respect *E. coli* codon frequency distribution. The encoded fusion protein sequence is SEQ ID NO:35.

- 25    Tables of *E. coli* codon usage used for codon optimisation for the above-mentioned constructs is given in “Codon usage in regulatory genes in *E. coli* does not reflect selection for rare codons”, Sharp P. M., Wen-Hsiung L., Nucleic Acids Research, 14, 7737-7749 (1986).

Constructs 1 and 2 are cloned in pMG1 plasmid. Plasmid pMG1 is a derivative of pMG27N. Plasmid pMG27N is a pBR322-derived expression vector which contains the P<sub>L</sub> promoter, an N utilization site (to relieve transcriptional polarity in the presence of N protein) and the cII ribosome binding site including the cII translation initiation codon incorporated in an Nde I restriction site (Gross *et al.* 1985, Mol.&Cell.Biol. 5:1015).  
 5 Plasmid pMG1 has been constructed by inserting the 81 first codons of the NS1 coding region from influenza strain A/PR/8/34 cleaved from plasmid pAS1ΔEH/801 (Young *et al.* 1983. *Proc.Natl.Acad.Sci.USA* 80:6105) by BamH I and Nco I into pMG27N digested by BamH I and Sac I. A synthetic DNA linker was introduced between the Nco I and the  
 10 Sac I sites. A schematic representation of pMG1 construction is shown in Figure 16.

Construct 3 to 5 are cloned in a commercial pET24b(+) plasmid from Novagen (Madison, WI).

Constructs 1 to 5 are transformed in the AR58 *E. coli* strain, which is a cryptic λ lysogen derived from N99 that is *gal* E::Tn 10,Δ-8(*chlD-pgl*),Δ-H1(*cro-chlA*),N<sup>+</sup>, and  
 15 cI857 (*Proc.Natl.Acad.Sci.USA* vol82, pp.88-92, January 1985 Biochemistry).

When the recombinant AR58 strains are available, the recombinant product is characterized by the evaluation of the level of expression and the prediction of further solubility of the protein by analysis of the behavior in the crude extract. After growth on appropriate culture medium and induction of the recombinant protein expression, total  
 20 extracts are analyzed by SDS-PAGE. The recombinant proteins are visualised in stained gels and identified by Western blot analysis using specific antibodies.

#### Expression of the recombinant protein from constructs 1 to 5:

Bacteria was grown in LB medium + 50 µg/ml Kan at 30°C. When the culture  
 25 reached OD = 0.5 (620nm), the culture was heated up to 39°C, after 5 hours of induction by ITPG, cells were harvested. The cell extract was prepared as follows: cells were resuspended in PBS buffer, disrupted (French press, 3 times), and centrifuged 30 minutes at 14000t. More than 90% of the protein was found in the supernatant of the cell extract.

#### Purification of the recombinant protein from constructs 1 to 5.

The purification scheme follows a classical approach based on the presence of an His affinity tail in the recombinant protein. In a typical experiment, the disrupted cells

are filtered and the acellular extracts loaded onto an Ion Metal Affinity Chromatography (IMAC;  $\text{Ni}^{++}$ NTA from Qiagen) that will specifically retain the recombinant protein. The retained proteins are eluted by 0-500 mM imidazole gradient (possibly in presence of a detergent) in a phosphate buffer.

- 5           The supernatant from the harvested culture was denatured in 6M urea, 100mM  $\text{NaH}_2\text{PO}_4$ , 10mM Tris, pH 8, and loaded on a chromatographic column IMAC Qiagen NTA  $\text{Ni}^{++}$  under the following conditions:

Equilibration buffer:  $\text{NaH}_2\text{PO}_4$  100mM (PH 8) Tris 10mM Urea 6M.

Sample: supernatant in urea 6M, 100mM  $\text{NaH}_2\text{PO}_4$ , 10mM Tris

- 10   Wash buffers:

1)  $\text{NaH}_2\text{PO}_4$  100mM PH 8 Tris 10mM Urea 6 M Imidazole 25mM

2)  $\text{NaH}_2\text{PO}_4$  100mM (PH8) Tris 10mM Urea 6mM Imidazole 50mM

Elution buffer:  $\text{NaH}_2\text{PO}_4$  100mM (PH 5,5) Tris 10mM Urea 6M Imidazole 500mM

- 15           The eluted protein in 500mM imidazole + 6M urea is dialysed under the following conditions:

1. PBS PH 7,2 + sarkosyl 0.5% + 4M Urea

2. idem at 2M Urea 2 hours

- 20   3. idem at 0M Urea 2 hours

#### Recombinant expression in microbial hosts results:

Construct 1 (NS1-CASB7439-HIS fusion protein):

- 25           The unpurified cell extracts is run on a 12.5% SDS PAGE, and subsequently stained with Coomassie blue. A Western blot is also performed using a monoclonal antibody directed against the 80 aminoacids N terminal fragment of the NS1 protein in fusion with CASB7439 construct 1. The resulting gels (Figures 17 and 18) show that the protein is expressed and visible in the cell extract supernatant.

- 30           The final purified material is frozen and stored. The protein content was quantified using a Lowry protein assay (0.9 mg/1.2 ml). The purity was assessed by a 12.5% PAGE SDS stained with Coomassie blue, and the presence of the recombinant protein was checked by Western blot, using a monoclonal antibody directed against the

80 aminoacids N terminal fragment of the NS1 protein in fusion with CASB7439 construct 1 (Figure 19).

Constructs 3 to 5 (T7 Tag - CASB7439 - HIS fusion protein):

5        Several dilutions of 15 µl of purified material for each construction is run on a 12.5% PAGE SDS gel. Presence and semi-quantification of recombinant protein is done by Western blot using SB600 anti-peptide antibody (see section 6.2.2 below for antibody description). Results, shown in Figure 20, clearly indicate CASB7439 constructs that have optimized codons for their *E. coli* host (constructs 3 and 4) yield higher quantities of  
10 recombinant protein. Using modified version of CASB7439 cDNAs therefore increases CASB7439 recombinant expression yields.

**14.2 Expression of the present invention antigen in vaccination vectors**

The immunogenicity of the antigen of the present invention has been verified by  
15 immunizing rabbits and mice using various means of immunization. Indeed, immunization with CASB7439 forms, either peptide or recombinant protein could induce humoral immune response with the generation of specific antibodies against CASB7439 and/or could induce a CASB7439 specific cellular immune response. Additionally, *in vivo* delivery of CASB7439 protein using for instance, naked DNA in an appropriate  
20 vector encoding CASB7439 or fragments of CASB7439, CASB7439 gene delivered by a viral vector encoding CASB7439 or fragments of CASB7439, is equally appropriate to demonstrate CASB7439 immunogenicity.

Recombinant expression of CASB7439 in DNA vaccination vectors

25        Several vectors can be used to construct the vaccinating plasmid (for instance, pcDNA3.1 vector, pJB16 vector). The plasmid expression vector for CASB7439 (pJB76) was constructed through the recombination of pDOR207/CASB7439 with the destination vector pJB16 (pVR1012-Dest) by the LR reaction of the Gateway Cloning Technology. HEK293T cells were transfected in 6-well plate with pJB76 DNA (0.25 or 1 µg), or  
30 empty vector (pJB16; 1ug). Cells were harvested at 48 hours post-infection and cell lysates were analyzed by Western blot for expression of CASB7439 using rabbit anti-NS1-CASB7439-His polyclonal antibody (dilution 1:500, see section 6.2.1 for antibody description). As shown in Figure 21, a band at the size expected for CASB7439 is

observed at low level in fibroblasts transfected with 1 ug of CASB7439 DNA (pJB76). No band is observed using with empty vector (pJB16).

#### Recombinant expression of CASB7439 in a adenoviral live vector

5            Recombinant adenoviruses are effective vectors for gene-based vaccination because they are capable of eliciting humoral and cellular immune responses against the encoded antigen. Recombining CASB7439 by such an approach is recommended for further immunological validations. Recombinant adenovirus for CASB7439 (AdC7439) was constructed using a Lac/GW-1 expression cassette under control of the CMV  
10   promoter.

            A schematic representation of the recombinant expression vector is shown in Figure 22. In brief,  $2 \times 10^5$  human fibroblasts from donor D93 were infected by the recombinant adenovirus for CASB7439 (AdC7439) in 6-well plate at various MOI. Cells were harvested at 48 hours post-infection and cell lysates were analyzed by Western blot  
15   for expression of CASB7439 using rabbit anti-NS1-CASB7439-His polyclonal antibody (1:500). As shown in Figure 23, a band at the size expected for CASB7439 is observed at low level in fibroblasts infected at an MOI of 200 and 300 and at high level at MOI of 400 or 500. No expression is seen in uninfected fibroblasts.

#### 20   Example 15

##### **Real-time RT- PCR analysis of the candidate antigen in panels of tumour and preneoplastic tissues of lung**

            Real-time RT-PCR (U. Gibson. 1996. Genome Research: 6,996) is used to evaluate and compare mRNA transcript abundance of the candidate antigen in panels of  
25   tumour and preneoplastic tissues of lung from multiple patient samples, of normal tissues, of different normal and tumour cell lines of lung, and finally of lung tumours grown *in vivo* in SCID mice. This analysis is important to establish the lung tumour specificity of CASB7439 expression, which is an important criterion a good antigen candidate must fulfil.

30           Primers for real-time PCR amplification are designed with the Perkin-Elmer Primer Express software using default options for TaqMan amplification conditions.

            Human tumour explants from lung cancer patients are implanted subcutaneously in SCID mice. When tumours reach ~5% of the total mouse body weight they are excised



and a portion (~1mm<sup>3</sup>) is passaged into another set of SCID mice. This process can be repeated indefinitely. Real time PCR analysis was performed on matched pairs (early passage/late passage) of SCID-derived human tumours.

Total RNA is extracted using TriPure reagent (Boehringer) and DNase treatment  
 5 from snap frozen biopsies of preneoplastic lesions of lung, early stage (stages Ib or IIa or IIb) non small cell lung carcinoma (NSCLC), late stage (stages IIIa or IIIb or IV) NSCLC, and several normal tissues, or from normal and tumour cell lines or lung tumours grown *in vivo* in SCID mice. Quantification of total RNA is performed by spectrofluorimetry (VersaFluor, BioRad) using SybrII dye (Molecular Probes), and  
 10 cDNA is obtained from the quantified total RNA by reverse transcription using a poly-dT oligonucleotide and Expand (Roche) reverse transcriptase.

Real-time reactions are assembled according to standard PCR protocols using 2 ng of reverse transcribed mRNA for each reaction. SybrI dye (Molecular Probes) is added at a final dilution of 1/75000 for real-time detection. Amplification (40 cycles) and  
 15 real-time detection is performed in a Perkin-Elmer Biosystems PE7700 system using conventional instrument settings. Ct values are calculated using the PE7700 Sequence Detector software. Several Ct values are obtained for each samples: for the panel of normal tissue samples, a CtXY for each normal tissue XY is calculated. An another Ct (CtA) is also obtained on  $\beta$ -Actin gene, as an internal reference, for all of the samples.

20 As the efficiency of PCR amplification under the prevailing experimental conditions is close to the theoretical amplification efficiency,  $2^{(CtA-CtXY)}$  value is an estimate of the relative TAA transcript level of the sample, standardised with respect to Actin transcript level. A value of 1 thus suggests the candidate antigen and Actin have the same expression level.

25 Absolute levels of CASB7439 transcript, as assessed by quantitative RT-PCR, in preneoplastic lesions of lung, early and late stage lung cancers and normal tissues is shown in Figures 20 to 28. Comparison of CASB7439 transcript abundance in preneoplastic lesions of lung, early and late stage lung cancers to normal tissues is depicted in table 1. This clearly show CASB7439 transcript is over-expressed in a vast  
 30 majority of patients suffering from lung preneoplastic lesions and late stage lung cancer. Overexpression fold in both populations of patients is close to 5. Some overexpression at comparable levels is indeed also seen in an early stage lung cancer population of patients, but coverage of overexpression is more restricted than those of preneoplastic lesions and

late stage lung cancer populations (see Table 6). In conclusion, CASB7439 lung preneoplastic lesion and lung tumour specificity agrees with its use as a diagnostic and immunotherapeutic agent in a lung cancer context.

5           **Table 6: CASB7439 transcript overexpression coverages and levels in lung preneoplastic, early and late stage lung cancer patient populations as compared to several normal tissues.**

% of preneoplastic patients showing a CASB7439 overexpression	100%
Average overexpression fold in overexpressing preneoplastic patients	5.04
Median overexpression fold in overexpressing preneoplastic patients	4.78
% of early stage lung cancer patients showing a CASB7439 overexpression	33%
Average overexpression fold in overexpressing early stage lung cancer patients	4.47
Median overexpression fold in overexpressing early stage lung cancer patients	2.87
% of late stage lung cancer patients showing a CASB7439 overexpression	83%
Average overexpression fold in overexpressing late stage lung cancer patients	4.69
Median overexpression fold in overexpressing late stage lung cancer patients	3.21

10    Example 16– Northern-Southern blot analysis

Limited amounts of mixed tumour and normal lung cDNA are amplified by Advantage PCR (see above). Messenger RNA from multiple normal tissues is also amplified using the same procedure. The amplified cDNA (1 µg) is electrophoresed on a 1.2% agarose gel and transferred onto a nylon membrane. The membrane is hybridised  
 15 (AlkPhos Direct System) with a probe prepared using a fragment of the candidate TAA cDNA. Northern-Southern analysis provides information on transcript size, presence of splice variants and transcript abundance in tumour and normal tissues.

Example 17

20    **17.1 Expression and purification of tumour-specific antigens**

### 17.1.1 Expression of the present invention antigen in microbial hosts.

Expression in microbial hosts, or alternatively *in vitro* transcription/translation, is used to produce the antigen of the invention for vaccine purposes and to produce protein fragments or whole protein for rapid purification and generation of antibodies needed for characterization of the naturally expressed protein by western blot, immunohistochemistry or for follow-up of purification.

Recombinant proteins may be expressed in two microbial hosts, *E. coli* and in yeast (such as *Saccharomyces cerevisiae* or *Pichia pastoris*). This allows the selection of the expression system with the best features for this particular antigen production. In general, the recombinant antigen will be expressed in *E. coli* and the reagent protein expressed in yeast.

The expression strategy first involves the design of the primary structure of the recombinant antigen. In general an expression fusion partner (EFP) is placed at the N terminal extremity to improve levels of expression that could also include a region useful for modulating the immunogenic properties of the antigen, an immune fusion partner (IFP). In addition, an affinity fusion partner (AFP) useful for facilitating further purification is included at the C-terminal end. Moreover, polynucleotide sequence of the coding region of the antigen can also be modified to optimise its expression. Using a codon usage that is adapted to the chosen recombinant expression host can dramatically increase antigen expression yields.

A comparative evaluation of the different versions of the expressed antigen will allow the selection of the most promising candidate that is to be used for further purification and immunological evaluation.

Several constructs underwent comparative evaluation: For rapid expression and purification as well as generation of antibodies against CASB7439, it is proposed to generate in *E. coli* a full length CASB7439 protein with *Influenza* protein NS1 as EFP and a histidine tail as AFP.

Five constructs have been designed as presented in Example 14:

**Construct 1:** Full length wild type CASB7439 cDNA in fusion with the N terminal fragment (the first 80 amino acids) of *Influenza* protein NS1 coding cDNA as EFP and

with a histidine tail coding cDNA as an AFP (SEQ ID NO:8). The encoded fusion protein sequence is SEQ ID NO:10. The CASB7439 protein design is shown in Figure 29.

**Construct 2:** Full length mutated CASB7439 cDNA in fusion with the N terminal  
 5 fragment of *Influenza* protein NS1 coding cDNA as EFP and with a histidine tail coding  
 cDNA as an AFP (SEQ ID NO: 9). This construct was designed in order to comprise the  
 first 50 codons of native CASB7439 cDNA replaced by codons specific of the *E. coli*  
 codon usage, to enhance expression potential of CASB7439 in its *E. coli* host. Only  
 CASB7439 codons that are known to be problematic in *E. coli* host by the man skilled in  
 10 the art were replaced by an *E. coli* codon. For each CASB7439 problematic codon, the *E.*  
*coli* codon is chosen to encode for the same amino acid and to have a low GC content.  
 The encoded fusion protein sequence is SEQ ID NO:10.

**Construct 3:** Full length wild type CASB7439 cDNA in fusion with a 11 amino acids  
 15 T7•Tag® (Novagen, Madison, WI) coding cDNA as EFP and with a histidine tail coding  
 cDNA as an AFP (SEQ ID NO:34). The encoded fusion protein is SEQ ID NO:35.

**Construct 4:** Full length mutated CASB7439 cDNA in fusion with a 11 amino acids  
 T7•Tag® (Novagen, Madison, WI) coding cDNA as EFP and with a histidine tail coding  
 20 cDNA as an AFP (SEQ ID NO:36). The first 50 codons of native CASB7439 cDNA  
 replaced by codons specific of the *E. coli* codon usage. The same substitutions as those  
 performed in construct 2 have been carried out in Construct 4. The encoded fusion protein  
 sequence is SEQ ID NO:35.

**Construct 5:** Full length mutated CASB7439 cDNA is fusion with a 11 amino acids  
 T7•Tag® (Novagen, Madison, WI) coding cDNA as EFP and a histine tail coding cDNA  
 as AFP (SEQ ID NO:37). This construct also has the first 50 codons of native CASB7439  
 cDNA replaced by codons specific of the *E. coli* codon usage. All CASB7439 codons are  
 modified in this construct to have specific *E. coli* codons and to respect *E.coli* codon  
 30 frequency distribution. The encoded fusion protein sequence is SEQ ID NO:35.

Tables of *E. coli* codon usage used for codon optimisation for the above-  
 mentioned constructs is given in “Codon usage in regulatory genes in *E. coli* does nor

reflect selection for rare codons”, Sharp P. M., Wen-Hsiung L., *Nucleic Acids Research*, 14, 7737-7749 (1986).

Constructs 1 and 2 are cloned in pMG1 plasmid. Plasmid pMG1 is a derivative of pMG27N. Plasmid pMG27N is a pBR322-derived expression vector which contains the PL promoter, an N utilization site (to relieve transcriptional polarity in the presence of N protein) and the cII ribosome binding site including the cII translation initiation codon incorporated in an Nde I restriction site (Gross *et al.* 1985, *Mol.&Cell.Biol.* 5:1015). Plasmid pMG1 has been constructed by inserting the 81 first codons of the NS1 coding region from influenza strain A/PR/8/34 cleaved from plasmid pAS1ΔEH/801 (Young *et al.* 1983. *Proc.Natl.Acad.Sci.USA* 80:6105) by BamH I and Nco I into pMG27N digested by BamH I and Sac I. A synthetic DNA linker was introduced between the Nco I and the Sac I sites. A schematic representation of pMG1 construction is shown in Figure 30.

Construct 3 to 5 are cloned in a commercial pET24b(+) plasmid from Novagen (Madison, WI).

Constructs 1 to 5 are transformed in the AR58 *E. coli* strain, which is a cryptic  $\lambda$  lysogen derived from N99 that is gal E::Tn 10,Δ-8(chlD-pgl),Δ-H1(cro-chlA),N+, and cI857 (*Proc.Natl.Acad.Sci.USA* vol82, pp.88-92, January 1985 *Biochemistry*).

When the recombinant AR58 strains are available, the recombinant product is characterized by the evaluation of the level of expression and the prediction of further solubility of the protein by analysis of the behavior in the crude extract. After growth on appropriate culture medium and induction of the recombinant protein expression, total extracts are analyzed by SDS-PAGE. The recombinant proteins are visualised in stained gels and identified by Western blot analysis using specific antibodies.

#### Expression of the recombinant protein from constructs 1 to 5:

Bacteria was grown in LB medium + 50 µg/ml Kan at 30°C. When the culture reached OD = 0.5 (620nm), the culture was heated up to 39°C, after 5 hours of induction by ITPG, cells were harvested. The cell extract was prepared as follows: cells were resuspended in PBS buffer, disrupted (French press, 3 times), and centrifuged 30 minutes at 14000t. More than 90% of the protein was found in the supernatant of the cell extract.

Purification of the recombinant protein from constructs 1 to 5.

The purification scheme follows a classical approach based on the presence of an His affinity tail in the recombinant protein. In a typical experiment the disrupted cells are filtered and the acellular extracts loaded onto an Ion Metal Affinity Chromatography (IMAC; Ni<sup>++</sup>NTA from Qiagen) that will specifically retain the recombinant protein.

The retained proteins are eluted by 0-500 mM imidazole gradient (possibly in presence of a detergent) in a phosphate buffer.

The supernatant from the harvested culture was denatured in 6M urea, 100mM NaH<sub>2</sub>PO<sub>4</sub>, 10mM Tris, pH 8, and loaded on a chromatographic column IMAC Qiagen

NTA Ni<sup>++</sup> under the following conditions:

Equilibration buffer: NaH<sub>2</sub>PO<sub>4</sub> 100mM (PH 8) Tris 10mM Urea 6M.

Sample: supernatant in urea 6M, 100mM NaH<sub>2</sub>PO<sub>4</sub>, 10mM Tris

Wash buffers:

1) NaH<sub>2</sub>PO<sub>4</sub> 100mM PH 8 Tris 10mM Urea 6 M Imidazole 25mM

2) NaH<sub>2</sub>PO<sub>4</sub> 100mM (PH8) Tris 10mM Urea 6mM Imidazole 50mM

Elution buffer: NaH<sub>2</sub>PO<sub>4</sub> 100mM (PH 5,5) Tris 10mM Urea 6M Imidazole 500mM

The eluted protein in 500mM imidazole + 6M urea is dialysed under the following conditions:

PBS PH 7,2 + sarkosyl 0.5% + 4M Urea

idem at 2M Urea 2 hours

idem at 0M Urea 2 hours

### Recombinant expression in microbial hosts results:

Construct 1 (NS1-CASB7439-HIS fusion protein):

The unpurified cell extracts is run on a 12.5% SDS PAGE, and subsequently stained with Coomassie blue. A Western blot is also performed using a monoclonal antibody directed against the 80 aminoacids N terminal fragment of the NS1 protein in fusion with CASB7439 construct 1. The resulting gels (Figures 17 and 18) show that the protein is expressed and visible in the cell extract supernatant.

The final purified material is frozen and stored. The protein content was quantified using a Lowry protein assay (0.9 mg/1.2 mL). The purity was assessed by a

12.5% PAGE SDS stained with Coomassie blue, and the presence of the recombinant protein was checked by Western blot, using a monoclonal antibody directed against the 80 aminoacids N terminal fragment of the NS1 protein in fusion with CASB7439 construct 1 (Figure 19).

5

Constructs 3 to 5 (T7 Tag - CASB7439 - HIS fusion protein):

Several dilutions of 15 µl of purified material for each construction is run on a 12.5% PAGE SDS gel. Presence and semi-quantification of recombinant protein is done by Western blot using SB600 anti-peptide antibody (see section 17.2.2 below for antibody description). Results, shown in Figure 31, clearly indicate CASB7439 constructs that have optimized codons for their *E. coli* host (constructs 3 and 4) yield higher quantities of recombinant protein. Using modified version of CASB7439 cDNAs therefore increases CASB7439 recombinant expression yields.

15 17.1.2 Expression of the present invention antigen in vaccination vectors

The immunogenicity of the antigen of the present invention has been verified by immunizing rabbits and mice using various means of immunization. Indeed, immunization with CASB7439 forms, either peptide or recombinant protein could induce humoral immune response with the generation of specific antibodies against CASB7439 and/or could induce a CASB7439 specific cellular immune response. Additionally, *in vivo* delivery of CASB7439 protein using for instance, naked DNA in an appropriate vector encoding CASB7439 or fragments of CASB7439, CASB7439 gene delivered by a viral vector encoding CASB7439 or fragments of CASB7439, is equally appropriate to demonstrate CASB7439 immunogenicity.

25

Recombinant expression of CASB7439 in DNA vaccination vectors

Several vectors can be used to construct the vaccinating plasmid (for instance, pcDNA3.1 vector, pJB16 vector). The plasmid expression vector for CASB7439 is constructed through the recombination of a CASB7439 recombinant plasmid with a destination vaccinating plasmid such as pcDNA3.1 or pJB16 by the LR reaction of the Gateway Cloning Technology. HEK293T cells are transfected in 6-well plate with recombinant vaccinating plasmid DNA (0.25 or 1 µg), or empty vector (1 µg). Cells were harvested at 48 hours post-infection and cell lysates were analyzed by Western blot for

30

expression of CASB7439 using rabbit anti-NS1-CASB7439-His polyclonal antibody (dilution 1:500, see section 6.2.1 for antibody description).

#### Recombinant expression of CASB7439 in a adenoviral live vector

- 5            Recombinant adenoviruses are effective vectors for gene-based vaccination because they are capable of eliciting humoral and cellular immune responses against the encoded antigen. Recombining CASB7439 by such an approach is recommended for further immunological validations. Recombinant adenovirus for CASB7439 was constructed using a Lac/GW-1 expression cassette under control of the CMV promoter.
- 10    In brief,  $2 \times 10^5$  human fibroblasts were infected by the CASB7439 recombinant adenovirus in 6-well plate at various MOI. Cells were harvested at 48 hours post-infection and cell lysates were analyzed by Western blot for expression of CASB7439 using rabbit anti-NS1-CASB7439-His polyclonal antibody (1:500).

#### 15    **17.2 Antibody production and immunohistochemistry**

Small amounts of relatively purified protein can be used to generate immunological tools in order to

- a) detect the expression by immunohistochemistry in normal or cancer tissue sections;
  - b) detect the expression, and to follow the protein during the purification process
- 20    (ELISA/ Western Blot); or
- c) characterize/ quantify the purified protein (ELISA).

##### 17.2.1 Polyclonal antibodies:

##### Immunisation

- 25            Rabbits were immunized, intramuscularly (I.M.), 3 times at 3 weeks intervals with 100µg of protein, formulated in the adjuvant 3D-MPL/QS21. Three weeks after each immunization a blood sample was taken and the antibody titer estimated in the serum by ELISA using the protein as coating antigen following a standard protocol.

##### 30    ELISA

96 well microplates (maxisorb Nunc) were coated with 5µg of protein overnight at 4°C. After 1 hour saturation at 37°C with PBS NCS 1%, serial dilution of the rabbit sera is added for 1H 30 at 37°C (starting at 1/10). After 3 washings in PBS Tween, anti rabbit



biotinylated anti serum (Amersham ) is added (1/5000). Plates are washed and  
 peroxydase coupled streptavidin (1/5000) is added for 30 minutes at 37°C. After  
 washing, 50µl TMB (BioRad) is added for 7 minutes and the reaction then stopped with  
 H<sub>2</sub>SO<sub>4</sub> 0.2M. The OD can be measured at 450 nm and midpoint dilutions calculated by  
 5 SoftmaxPro.

### 17.2.2 Anti-peptide antibodies.

Besides being highly tumour specific, the second critical criterion for a candidate  
 vaccine evaluation is its immunogenicity. One way of assessing the immunogenicity of a  
 10 protein is to immunize naïve animals with synthetic peptides derived from the antigen  
 sequence, and which reproduce natural epitopes. The generated anti-peptide antibodies  
 will then tend to recognise the native antigen, therefore showing a specific immune  
 response can be raised against the candidate vaccine antigen.

Because of the very nature of the immune response, a classical dose range  
 15 analysis is replaced by repeated immunizations, allowing comparison of antibody titers  
 before immunization (control), and after cumulative injections.

Two peptides were selected from CASB7439 antigen sequence for their  
 immunogenic potential: peptides spanning from amino-acids 1-14 (peptide 1) and amino-  
 acids 157-172 (peptide 2). Two rabbits were immunized with each peptide, rabbits SB598  
 20 and SB599 with peptide 1 and rabbits SB600 and SB601 with peptide 2.

The selected peptides were conjugated to a carrier protein (KLH). Rabbits were  
 intramuscularly immunized with CASB7439 peptide, 3 times at 3 to 4 weeks intervals  
 with 200 µg of conjugate formulated with Freund's adjuvant. Four weeks after the second  
 immunization (PP) and four weeks after the third immunization (GP), blood samples  
 25 were taken, and the CASB7439 specific antibody titers were estimated in the serum by  
 ELISA. Thus, a dose range of 0, 200 and 400 µg of the antigen conjugate is reproduced.

ELISAs were done in triplicate for each immunizing peptide and rabbit serum,  
 and performed as follows: 96 well microplates were coated at 4°C during 16 hours with  
 either 100 ng of CASB7439 peptide or 100 ng of KLH as coating antigens. After 2-hour  
 30 saturation at 25°C with BSA (1 mg/ml), serial dilution of the rabbit sera was added for 2  
 hours at 25°C (starting at dilution 1/100). Anti rabbit antibody, conjugated with universal-  
 HRP, was then added at dilution 1/1000 for 2 hours at 25°C as secondary antibody. Plates

were washed and OPD (0.4 mg/ml) is added for 30 minutes at 25°C. Reaction was stopped with H<sub>2</sub>SO<sub>4</sub> 4M, and OD measured at 492 nm.

CASB7439 peptide immunization ELISA read-out results are shown in tables 7 to 10 and Figures 32 to 35. Results clearly show an antibody immune response against distinct CASB7439 synthetic peptide can be raised in several rabbits in a dose-dependant manner. This suggests CASB7439 candidate antigen is indeed immunogenic and, when properly formulated with adjuvant as a vaccine, CASB7439 vaccine is able to induce a strong and specific antibody immune response.

Table 7: CASB7439 peptide 1 immunization of rabbit SB598: ELISA read-outs.

Dilution	CASB7439 Peptide									KLH Carrier		
	PPI (preimmune) Replicate:			PP Replicate:			GP Replicate:			PPI	PP	GP
	1	2	3	1	2	3	1	2	3			
100	0.096	0.116	0.123	2.734	2.753	2.821	3.305	3.305	3.306	0.137	3.532	3.532
300	0.066	0.076	0.082	1.622	1.582	1.535	2.813	2.79	2.85	0.084	3.405	3.445
900	0.056	0.064	0.067	0.587	0.582	0.532	1.336	1.221	1.333	0.078	2.899	2.55
2700	0.059	0.067	0.059	0.243	0.216	0.183	0.592	0.435	0.472	0.064	1.548	1.246
8100	0.121	0.057	0.068	0.113	0.099	0.086	0.23	0.163	0.167	0.061	0.623	0.443
24300	0.051	0.056	0.052	0.09	0.072	0.065	0.124	0.096	0.094	0.063	0.269	0.2
72900	0.055	0.058	0.056	0.061	0.08	0.057	0.07	0.065	0.17			
218700	0.056	0.052	0.055	0.065	0.056	0.054	0.057	0.059	0.057			

Table 8: CASB7439 peptide 1 immunization of rabbit SB599: ELISA read-outs.

Dilution	CASB7439 Peptide									KLH Carrier		
	PPI (preimmune) Replicate:			PP Replicate:			GP Replicate:			PPI	PP	GP
	1	2	3	1	2	3	1	2	3			
100	0.068	0.053	0.065	3.172	3.347	3.294	3.354	3.248	3.283	0.105	3.582	3.493
300	0.047	0.057	0.06	2.468	2.399	2.437	2.748	2.855	2.848	0.082	3.415	3.391
900	0.053	0.057	0.058	1.315	1.19	1.27	1.603	1.45	1.43	0.07	2.941	2.763
2700	0.055	0.06	0.059	0.598	0.472	0.493	0.669	0.551	0.528	0.067	1.709	1.526
8100	0.054	0.055	0.056	0.207	0.164	0.171	0.214	0.185	0.178	0.065	0.625	0.573
24300	0.053	0.054	0.056	0.098	0.087	0.092	0.104	0.091	0.092	0.067	0.263	0.249
72900	0.057	0.057	0.059	0.064	0.063	0.061	0.08	0.067	0.067			
218700	0.065	0.06	0.061	0.057	0.059	0.056	0.056	0.056	0.058			

Table 9: CASB7439 peptide 2 immunization of rabbit SB600: ELISA read-outs.

Dilution	CASB7439 Peptide									KLH Carrier		
	PPI (preimmune)			PP			GP			PPI	PP	GP
	Replicate:			Replicate:			Replicate:					
	1	2	3	1	2	3	1	2	3			
100	0.078	0.08	0.074	3.195	3.225	3.179	3.253	3.236	3.256	0.116	3.545	3.573
300	0.063	0.073	0.064	2.758	2.823	2.874	3.216	3.229	3.251	0.08	3.479	3.502
900	0.068	0.063	0.062	1.606	1.536	1.699	2.585	2.585	2.581	0.081	2.485	2.92
2700	0.066	0.064	0.065	0.843	0.746	0.704	1.361	1.44	1.328	0.074	1.225	2.026
8100	0.064	0.068	0.064	0.48	0.392	0.401	0.778	0.813	0.722	0.068	0.721	1.067
24300	0.063	0.069	0.062	0.21	0.163	0.224	0.283	0.299	0.247	0.069	0.237	0.405
72900	0.068	0.067	0.066	0.114	0.101	0.098	0.131	0.135	0.115			
218700	0.076	0.065	0.063	0.078	0.073	0.071	0.08	0.08	0.082			

Table 10: CASB7439 peptide 2 immunization of rabbit SB601: ELISA read-outs.

Dilution	CASB7439 Peptide									KLH Carrier		
	PPI (preimmune)			PP			GP			PPI	PP	GP
	Replicate:			Replicate:			Replicate:					
	1	2	3	1	2	3	1	2	3			
100	0.069	0.072	0.08	2.443	2.536	2.57	2.973	3.019	3.077	0.117	3.497	3.507
300	0.064	0.065	0.065	1.427	1.469	1.592	2.366	2.381	2.44	0.077	3.156	3.328
900	0.064	0.063	0.065	0.704	0.699	0.751	1.343	1.368	1.314	0.068	2.416	2.695
2700	0.062	0.064	0.065	0.302	0.302	0.298	0.76	0.589	0.532	0.078	1.106	1.29
8100	0.062	0.065	0.065	0.126	0.323	0.129	0.221	0.218	0.205	0.183	0.49	0.484
24300	0.061	0.064	0.06	0.075	0.078	0.079	0.111	0.1	0.09	0.095	0.181	0.176
72900	0.063	0.065	0.063	0.072	0.071	0.073	0.102	0.08	0.074			
218700	0.064	0.062	0.065	0.069	0.067	0.074	0.069	0.068	0.072			

### 17.2.3 Monoclonal antibodies:

#### Immunisation

5 BALB/c mice are immunized 3 times at 3 week intervals with 5 µg of purified protein. Bleedings are performed 14 days post II and 1 week post 3. The sera are tested by Elisa on purified protein used as coated antigen. Based on these results (midpoint dilution >10000) one mouse is selected for fusion.

#### Fusion/ HATselection

Spleen cells are fused with the SP2/0 myeloma according to a standard protocol using PEG 40% and DMSO 5%. Cells are then seeded in 96 well plates 2.5 x10<sup>4</sup> - 10<sup>5</sup> cells/well and resistant clones will be selected in HAT medium. The supernatant of these hybridomas will be tested for their content in specific antibodies and when positive, will

be submitted to 2 cycles of limited dilution. After 2 rounds of screening, 3 hybridomas will be chosen for ascitis production.

### 17.2.3 Immunohistochemistry

- 5           When antibodies are available, immunostaining is performed on normal or cancer tissue sections, in order to determine:
- the level of expression of the antigen of the invention in cancer relative to normal tissue
- or the proportion of cancer of a certain type expressing the antigen
- if other cancer types also express the antigen
- 10       the proportion of cells expressing the antigen in a cancer tissue

### Tissue sample preparation

- After dissection, the tissue sample is mounted on a cork disk in OCT compound and rapidly frozen in isopentane previously super cooled in liquid nitrogen (-160°C). The
- 15       block will then be conserved at -70°C until use. 7-10µm sections will be realised in a cryostat chamber (-20, -30°C).

### Staining

- Tissue sections are dried for 5 minutes at room temperature ("RT"), fixed in
- 20       acetone for 10 minutes at RT, dried again, and saturated with PBS 0.5% BSA 5% serum. After 30 minutes at RT either a direct or indirect staining is performed using antigen specific antibodies. A direct staining leads to a better specificity but a less intense staining whilst an indirect staining leads to a more intense but less specific staining.

- 25       17.3 Analysis of human cellular immune responses to the antigen of the invention

- The immunological relevance of the antigen of the invention can be assessed by *in vitro* priming of human T cells. All T cell lymphocyte lines and dendritic cells are derived from PBMCs (peripheral blood mononuclear cells) of healthy donors (HLA-A2 subtype). An HLA-A2.1/Kb transgenic mouse model is also used for screening of HLA-
- 30       A2.1 peptides.

CD8+ T-cell response

Newly discovered antigen-specific CD8+ T cell lines are raised and maintained by weekly *in vitro* stimulation. The lytic activity and the  $\gamma$ -IFN production of the CD8+ lines in response to the antigen or antigen derived-peptides is tested using standard  
 5 procedures.

Two strategies to raise the CD8+ T cell lines are used: a peptide-based approach and a whole gene-based approach. Both approaches require the full-length cDNA of the newly discovered antigen in the correct reading frame to be either cloned in an appropriate delivery system or to be used to predict the sequence of HLA binding  
 10 peptides.

Peptide-based approach

Briefly, transgenic mice are immunized with adjuvanted HLA-A2 peptides, those unable to induce a CD8+ response (as defined by an efficient lysis of peptide-pulsed  
 15 autologous spleen cells) will be further analyzed in the human system.

Human dendritic cells (cultured according to Romani *et al.*) will be pulsed with peptides and used to stimulate CD8+ -sorted T cells (by FACS). After several weekly stimulations, the CD8+ lines will be first tested on peptide-pulsed autologous BLCL (EBV-B transformed cell lines). To verify the proper *in vivo* processing of the peptide,  
 20 the CD8+ lines will be tested on cDNA-transfected tumour cells (HLA-A2 transfected LnCaP, Skov3 or CAMA tumour cells).

Whole gene-based approach

CD8+ T cell lines will be primed and stimulated with either gene-gun transfected  
 25 dendritic cells, retrovirally transduced B7.1-transfected fibroblasts, recombinant pox virus or adenovirus infected dendritic cells. Virus infected cells are very efficient to present antigenic peptides since the antigen is expressed at high level but can only be used once to avoid the over-growth of viral T cells lines.

After alternated stimulations, the CD8+ lines are tested on cDNA-transfected  
 30 tumour cells as indicated above. Peptide specificity and identity is determined to confirm the immunological validation.

CD4+ T-cell response

Similarly, the CD4+ T-cell immune response can also be assessed. Generation of specific CD4+ T-cells is made using dendritic cells loaded with recombinant purified protein or peptides to stimulate the T-cells.

5           The above description fully discloses how to make and use the present invention. However, this invention is not limited to the particular embodiments described hereinabove, but includes all modification thereof within the scope of the appended claims and their equivalents. Those skilled in the art will recognize through routine experimentation that various changes and modifications can be made without departing  
10       from the scope of this invention. The various references to journals, patents and other patent applications that are cited herein are incorporated by reference herein as though fully set forth.

## SEQUENCE INFORMATION

SEQ ID NO:1

5 GTACCTTGCTTTGGGGGCGCACTAAGTACCTGCCGGGAGCAGGGGGCGCACCGGGAACTCGCAGATTTTCGCC  
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 15 CAAGAAGCTGAGCAAGGTGGAGACGCTGCGCTCAGCCGTGGAGTACATCCGCGCGCTGCAGCGCTGCTGGC  
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30 SEQ ID NO:2

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35 SEQ ID NO:3

MSAPAARSASGAEAHRSRALSSPLTSWRSRVARAPQDSARLRSRCRPTSRRNAGSRAPSCPRGPGTKKRGRA  
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 SPSSPASRGCAHCAALRPLRRLRSLRWPVAAAGCSATVPGTRVSAGQSRQGRGAQGARTWAVCRRPSRL  
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SEQ ID NO:4

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 5 CCGTGCGGCACGTGCTGCCGCAGCGCCTGGAAGCCCAAGTTCACCAGCTTCACGCGGTTGCGCTCGCGCTCA  
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 AACAGTTCGGGGACGCGGGTCTCCGCCGGGACGCGAGCCGACAGGGACGGGGGCGCAGGGGGCGCGGAC  
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 GTGCAGGCAGAGGAACCGGAGGCGACGGGAAAACCTGTGGCGCCCCAAGGGGGCTTCTGGCACGGCGCCGCC  
 10 AGGCAACTCCCCAGGGACGCGTCTTAGGTGCTCTGGAGCCCGGGATAGGAGGCCTAGTGGTGGCAGGCCG  
 TACGCGCCAGGGAGCGTGGGACGCTCGTGTCCCGCGCGTGGCGCCGGACTCTCCCAGGTCTCCGCAGGCGCG  
 GCGCAGGCGGCTGGTTTTTAAATGTATAGATAACCCCTCCTCCGCGCCGCCGCGCTCGCTTTCTCACGCCCT  
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 CGTGCGCATGTCCCCCTAGCCCATCCCCCGGACGAGTCCACAGATCCCCAGTGCGCCCAACTGGCGAAATC  
 15 TGCAGTTCCCGGTGCGCCCCCTGCTCCCGGCAGGTACTTAGTGCGCCCCCAAGCAAGGTAC

SEQ ID NO: 7

MCRKWILCALRKSSPLRKNLQVLVPPAPLQSRSCGEGRRRRKPPALMGPAFSPFPPRHWSGWAGRTRRRRRRC  
 GGWWVGPRLAGGARARSTLAGFPGDEARRPVRSGFRGLRLIRSRALSSPLTSWRSRVARAPQDSARLRSRC  
 20 RPTSRRNAGSRAPSCPRGPGTKKRGRARRRPGWSLAARGAQTAAARPAASALPPARCARRRRARPAGAAARGCT  
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 S

25 SEQ ID NO: 8

ATGGATCCAAACACTGTGTCAAGCTTTCAGGTAGATTGCTTTCTTTGGCATGTCCGCAAACGAGTTGCAGAC  
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 ACCCTCGGTCTGGACATCGAGACAGCCACACGTGCTGGAAAGCAGATAGTGGAGCGGATTCTGAAAGAAGAA  
 TCCGATGAGGCACTTAAAATGACCATGGACGGCGGCACACTGCCCAGGTCCGCGCCCCCTGCGCCCCCGTC  
 30 CCTGTGCGCTGCGCTGCCCCGGCGGAGACCCGCTCCCGGAACTGTTGCGCTGCAGCCGGCGGCGGCGACCG  
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 CTGGTGAACCTGGGCTTCCAGGCGCTGCGGCAGCACGTGCCGCACGGCGGCGCCAGCAAGAAGCTGAGCAAG  
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 35 ACCCCGGTCGCGCCTCGCCCTCCCGCGCTTCTTCGTCCCCGGGCCGCGGGGCGAGCTCGGAGCCCGGTCC  
 CCGGTTCCGCTACTCGTCGGACGACAGCGGCTGCGAAGGCGCGCTGAGTCTGCGGAGCGCGAGCTACTC  
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SEQ ID NO: 9

40 ATGGATCCAAACACTGTGTCAAGCTTTCAGGTAGATTGCTTTCTTTGGCATGTCCGCAAACGAGTTGCAGAC  
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 CCGGTTGGCTGCGCTGCCCCGTGCGGCTCCCGCGTCCCGGAACTGCTGCGCTGCAGCCGTGCGCGTCCCCG  
 45 GCCACCGCAGAGACCGGAGGCGGCGCAGCGGCCGTAGCGCGGCGCAATGAGCGCGAGCGCAACCGCGTGAAG

CTGGTGAACCTGGGCTTCCAGGCGCTGCGGCAGCACGTGCCGCACGGCGGCCAGCAAGAAGCTGAGCAAG  
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 CGAACGCGCTGGCGGGAGGGCTGAGGCCGAGGCCGTGCGGCCGTCTGCGCCCCGCGGGCCGCCAGGGACC  
 ACCCCGGTGCCTCGCCCTCCCGCGCTTCTTCGTCCCCGGGCGCGGGGCGAGCTCGGAGCCCGGCTCC  
 5 CCGCGTTCCGCCTACTCGTCGGACGACAGCGGCTGCGAAGGCGCGCTGAGTCTTGCAGGCGCGAGCTACTC  
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SEQ ID NO:10

MDPNTVSSFQVDCFLWHVRKRVADQELGDAPFLDRLRRDQKSLRGRGSTLGLDIETATRAGKQIVERILKEE  
 10 SDEALKMTMDGGTLPRSAPPAPPVPVPGCAARRRPASPELLRCSRRRRPATAETGGGAAAVARRNERERNRVK  
 LVNLGFQALRQHVPVPHGGASKKLSKVETLRSAYEYIRALQRLLAEHDAVRNALAGGLRPQAVRPSAPRGPPT  
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SEQ ID NO:11

MYSTAERSVSTLLSFLAPPCGTCCRSAPKPKFTSFTRLRSRLRRATAAAPPVSAVAGRRRLQRNSSGD  
 15 AGLRRAAQPTGTGGAGGADLGSVPPSIAPASTRPLQVPARRRKVQAEPEATGKTVPQGGFWHGAARQLPR  
 ARVLGRLEPGDRRPSGGRPYAPGSVGRSCPARAAGLSQVSAGAAQAAGF

SEQ ID NO:12

MEAHLDWYGVPLQEASDACPRESCSSALPEAREGANVHFPPHPVPREHFSCAAPELVAGAQGLNASLMDGG  
 20 ALPRLMPTSSGVAGACAARRRQASPELLRCSRRRRSGATEASSSSAAVARRNERERNRVKLVNLGFQALRQH  
 VPHGGANKKLSKVETLRSAYEYIRALQRLLAEHDAVRAALAGLLTPATPPSDECAQPSASPASASLSCAST  
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25 SEQ ID NO:13

GGCCGGAGCATGGAAGCACGTCAGCTAGGCCATGAACTGCACCCGGGAGGGGTGGGGGTGGAAGCGCACGGT  
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 GGCGGGGCGGAGGAGATTATCTATACCTTTTAAAAAAAAGGAGCCTCTTAGCCGCGTAAAGGAGACTTGGGG  
 AGCGCCTGACAGCACGCGCGGGACACGAGAGTACCACGCTTCCCTACTCTTTTCAGACCTTGACTGGTACGG  
 30 GGTCCCAGGACTGCAGGAGGCCAGCGACGCGTGCCTAGGGAGTCTTCGAGCAGTGCCCTGCCTGAGGCCCG  
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 CGTAGCAGGGGCCAGGGGCTGAATGCAAGCTTGATGGACGGCGCGCGCTGCCAGACTCATGCCACCTC  
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 35 CCGCGTAAAGCTGGTAAACTTGGGCTTCCAGGCGCTGCGGCAGCACGTGCCGCACGGCGGGCGCCAACAAGAA  
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 GCCCTCTGCCCTCCCTGCCAGCGGTCTCTGTCTGCGCCTCTACGTCTCCGTCCCGGACCTTGGGCTGCTC  
 TGAGCCTACCTCCCCGCGCTCCGCTACTCGTCCGAGGAAAGCAGCTGCGAGGGAGAGCTAAGCCCCGATGGA  
 40 GCAGGAGCTGCTTGACTTTTCCAGTTGGTTAGGGGGCTACTGA

SEQ ID NO:14

MESHFNWYGVPLQKASDACPRESCSSALPEAREGANVHFPPHPVPREHFSAGAPKPVAGAPALNASLMDGG  
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VPHGGANKKLSKVETLRSAYEYIRALQRLLAEHDAVRAALSGLLTPATRPSDVCTQPSASPASASLSCTST  
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SEQ ID NO:15

5 TTCACCCGGCTGCAAGCGCTAGGTGTACGGAGACCTGGCAGCTCTTGGGGCTTAAGGACTGAGCRCCAGAGC  
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TTCGGGATCTGCACTCGAGGACTTGTCTGAGGACGCATTAAGCTAAGCATCTGCTCGGAGCATGGAATCGCAC  
TTTAACTGGTACGGGGTCCCAAGGCTCCAGAAGGCTAGCGACGCGTGCCCTAGGGAATCCTGCAGCAGTGCC  
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10 GGCGCACCGAAACCCGTAGCGGGGGCCCCGGCGCTGAATGCAAGCTTGATGGACGGCGGCGCGCTGCCCAGA  
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15 CTGCTAGCAGAGCACGACGCGGTGCGTGCTGCGCTCTCTGGGGGTCTATTAACACCCGCTACTCGGCCGTCC  
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20 GCTGTCTAGTGCAGCCTGACCAAATGCCAAGTACTGACCTCTGCTCGGCCTCCACGCCGCGGAATGACATCT  
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CTTTTATCAATTAACCTGAACTGCTGGAGGACTCTGCTGAAAATATGAAGAATTATTTTTATACAAAGGAT  
CCTTAAGCTTGGAGCACATAAAGATGACCTCTGTCTCTACCCCCACTGTCTAGAACTTTCCAACCTGGCC  
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25 TGACGCTGATGTTTGGCCAGTGTGGGAACCCTGCTATTGCAAAGTGTACTATTCTATAAAAGTTGTTTTTCA  
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SEQ ID NO:16

KLVNLGFQAL

30

SEQ ID NO:17

ELLDFSSWL

SEQ ID NO:18

35 RLLAEHDAV

SEQ ID NO:19

KLVNLGFQA

40 SEQ ID NO:20

EYIRALQRL

SEQ ID NO:21

EYIRALQRL

45

# BC45300-1

SEQ ID NO:22  
AVRNALAGGL

5 SEQ ID NO:23  
SEPGSPRSAY

SEQ ID NO:24  
VETLRSAVEY

10 SEQ ID NO:25  
IRALQRLLA

SEQ ID NO:26  
LRPQAVRPS

15 SEQ ID NO:27  
LRQHVPHGG

SEQ ID NO:28  
20 LGFQALRQH

SEQ ID NO:29  
VRNALAGGL

25 SEQ ID NO:30  
YIRALQRL

SEQ ID NO:31  
LVNLGFQAL

30 SEQ ID NO:32  
VEYIRALQR

SEQ ID NO:33  
35 LLRCSRRRR

SEQ ID NO:34  
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40 AGCCGGCGGCGGCGACCGGCCACCGCAGAGACCGGAGGCGGCGCAGCGCCGTAGCGCGGCGCAATGAGCGC  
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CACTGA

SEQ ID NO:35

5 MASMTGGQQMGRDPMDDGGTLPRSAPPAPPVPGCAARRRPASPELLRCSRRRRPATAETGGGAAAVARRNER  
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RGPPGTTPVAASPSRASSSPGRGGSSEPGSPRSAYSSDDSGCEGALSPAERELLDFFSSWLGGYTSLEHHHHH  
H

10 SEQ ID NO:36

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15 AGCAAGAAGCTGAGCAAGGTGGAGACGCTGCGCTCAGCCGTGGAGTACATCCGCGCGCTGCAGCGCCTGCTG  
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CGCGGGCCGCCAGGGACCACCCCGGTGCGCCGCTCGCCCTCCCGCGCTTCTTCTGTCGCCGGCCGCGGGGGC  
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20 CACTGA

SEQ ID NO:37

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25 TCTCGTCGCAGACGTCCAGCTACCGCAGAGACCGGAGGCGGCGCAGCGGCCGTAGCGCGGCGCAATGAGCGC  
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30 AGCTCGGAGCCCGGTCCCCGCGTTCCGCC TACTCGTCGGACGACAGCGGCTGCGAAGGCGCGCTGAGTCCT  
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CACTGA